A BIOCHEMICAL INVESTIGATION OF THE N-METHYL-D-ASPARTATE RECEPTOR IN THE RAT CENTRAL NERVOUS SYSTEM

by

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DECLARATION

In accordance with the requirements of the University of Edinburgh regulation 3.4.7, this thesis has been composed by myself and the work presented herein is my own.

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Activation of the N-methyl-D-aspartate(NMDA) receptor is important in both physiological and pathological phenomena, including synaptogenesis, long-term potentiation, neuroexcitotoxicity and epilepsy. Although implicated in postnatal formation of synaptic connections, over-activation of the NMDA receptor in the neonate, as a result of say a hypoxic-ischaemic insult, can lead to neuronal damage. Antagonists for specific sites on the NMDA receptor complex may therefore prove to be novel therapeutic agents for preventing excitotoxic neuronal damage. I have therefore investigated the ontogeny of the NMDA receptor complex as well as modulation of the receptor in both adult and neonatal rat brain tissue.

Radioligand binding studies were performed using high-affinity NMDA receptor antagonists. [^{3}H]3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonoate ([^{3}H]CPP) and [^{3}H]D-2-amino-5-phosphonopentanoate([^{3}H]D-AP5), both competitive antagonists which bind to the NMDA neurotransmitter recognition site, and [^{3}H]dizocilpine a non-competitive antagonist which binds to a site within the lumen of the NMDA-associated ion channel, were used. Optimal experimental conditions were established for the binding of each ligand to membranes prepared from rat brain tissue. This allowed reliable and reproducible measurements to be made under different modulatory conditions. Using mature tissue the binding of [^{3}H]CPP was compared with that of [^{3}H]AP5 in the presence of compounds active at the glycine modulatory site including glycine, 7-chlorokynurenate and 3-amino-1-hydroxypyrrolid-2-one(HA-966). Differential effects were seen on the binding of each ligand. The findings provide further evidence for the hypothesis that the NMDA receptor exists in more than one conformational state, these being regulated by glycine.

[^{3}H]CPP and [^{3}H]dizocilpine were used to investigate the ontogeny of their respective binding sites. Each ligand bound to membranes prepared at various postnatal ages between birth and adulthood. The binding of both ligands was detectable at the earliest ages examined, increasing with postnatal age. [^{3}H]CPP binding reached a level equivalent to that seen in adult rats during the fourth week of life. [^{3}H]Dizocilpine binding was investigated in the presence and absence of L-glutamate and/or glycine to try and elucidate underlying modulatory changes resulting in alterations in postnatal binding. Binding was modulated by both amino acids at all ages tested. Specific binding under each condition reached adult equivalent levels at earlier ages than seen for [^{3}H]CPP binding. For both ligands the differences in binding between postnatal ages were due to an alteration in receptor density but not to an alteration in receptor affinity. The receptor density for [^{3}H]dizocilpine binding did not alter significantly between experimental conditions, thus confirming that only the receptor affinity is influenced by L-glutamate and/or glycine. As for adult tissue this was also true for immature tissue.

Clearly both binding sites, as well as the glycine modulatory site, are present and functionally coupled from an early stage of postnatal development. However, the neurotransmitter and the ion channel sites have different developmental time-courses. Postnatal development of the [^{3}H]CPP site does however have a similar profile to the postnatal development of L-glutamate levels as well as to the development of synaptic connections in rat brain. NMDA antagonists active at any of the three mentioned sites could therefore play an important role in the treatment of neonatal as well as adult excitotoxic damage caused by excessive activation of the NMDA receptor.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 HISTORY

A wealth of evidence suggests that acidic amino acids are important endogenous excitatory neurotransmitters in the mammalian central nervous system (CNS) although the actual identity of the excitatory amino acid (EAA) neurotransmitter(s) remains elusive. L-Glutamate and L-aspartate still remain the most likely candidates. Topical application of the amino acids L-glutamate and L-aspartate to the mammalian cerebral cortex was shown to cause convulsive behaviour in the mid 1950's (Hayashi, 1954). Curtis and Watkins, pioneers in the field of EAA research, later demonstrated that L-glutamate and other monoamino-dicarboxylic amino acids depolarised cat spinal neurones, resulting in the generation of action potentials (Curtis and Watkins, 1960). These initial findings have led to a great explosion of research in the EAA field, especially in the last decade. Specific receptors for the EAA neurotransmitter(s) have been identified in the mammalian CNS and physiological roles have been assigned to them (Watkins *et al*, 1990). The N-methyl-D-aspartate (NMDA) receptor is the most widely investigated EAA receptor. It has been implicated as having a number of physiological and pathological roles and interest in the NMDA receptor lies in the potential of NMDA receptor antagonists as therapeutically useful drugs in a wide range of neurological dysfunctions (Collingridge and Lester, 1989). Paradoxically non-NMDA receptors were cloned, and the molecular structure known, earlier than the NMDA receptor (Hollman *et al*, 1989; Masu *et al*, 1991; Keinanen *et al*, 1990). Recently however L-glutamate receptor subunits exhibiting typical NMDA receptor characteristics have been cloned (Moriyoshi *et al*, 1991; Meguro *et al*, 1992). Work can now proceed to determine the molecular mechanisms of both NMDA and non-NMDA receptors.

1.2 EAA NEUROTRANSMITTER CANDIDATES IN THE MAMMALIAN CNS

Neurotransmitter substances may be classified either as excitatory or inhibitory, depending on whether the substance causes a depolarisation or a hyperpolarisation of the neuronal membrane. Glycine and γ-amino-butyric acid (GABA) have been identified and characterised as the major inhibitory neurotransmitters in the adult mammalian CNS, whereas L-glutamate is thought to be the major excitatory neurotransmitter (Graham *et al*, 1967; Fagg and Foster, 1983; Fonnum, 1984). It has already been identified as the transmitter at the insect neuromuscular junction (Usherwood, 1981). Other excitatory amino acid neurotransmitter candidates in the mammalian CNS include L-aspartate, N-acetyl-L-aspartyl-L-glutamate (NAAG), quinolinate and the sulphur containing analogues of L-glutamate and L-asparate such as L-homocysteic acid (L-HCA) (Fig.1; Collingridge and Lester, 1989). To be classed as a neurotransmitter a substance must satisfy a number of specific criteria. These criteria were originally proposed for identification of peripheral transmitters but can also be generalised to the CNS, (Orrega, 1979). These criteria state that the transmitter must be present in presynaptic terminals with synthetic enzymes capable of synthesising the neurotransmitter from precursors. The transmitter should be released in sufficient quantities to elicit a postsynaptic response which can be mimicked by exogenous application of the substance. The transmitter candidate should also have a regional distribution in the CNS, with a mechanism for inactivation. It is difficult to prove that a candidate substance satisfies all the criteria but the potential excitatory amino acid candidates shall now be considered.

L-Glutamate

Most available evidence points to L-glutamate being the principle excitatory neurotransmitter candidate in the mammalian CNS. L-Glutamate has the highest concentration (10mM) of all amino acids in the CNS (Perry *et al*, 1971), but shows no differential regional distribution throughout the mammalian brain with the exception of

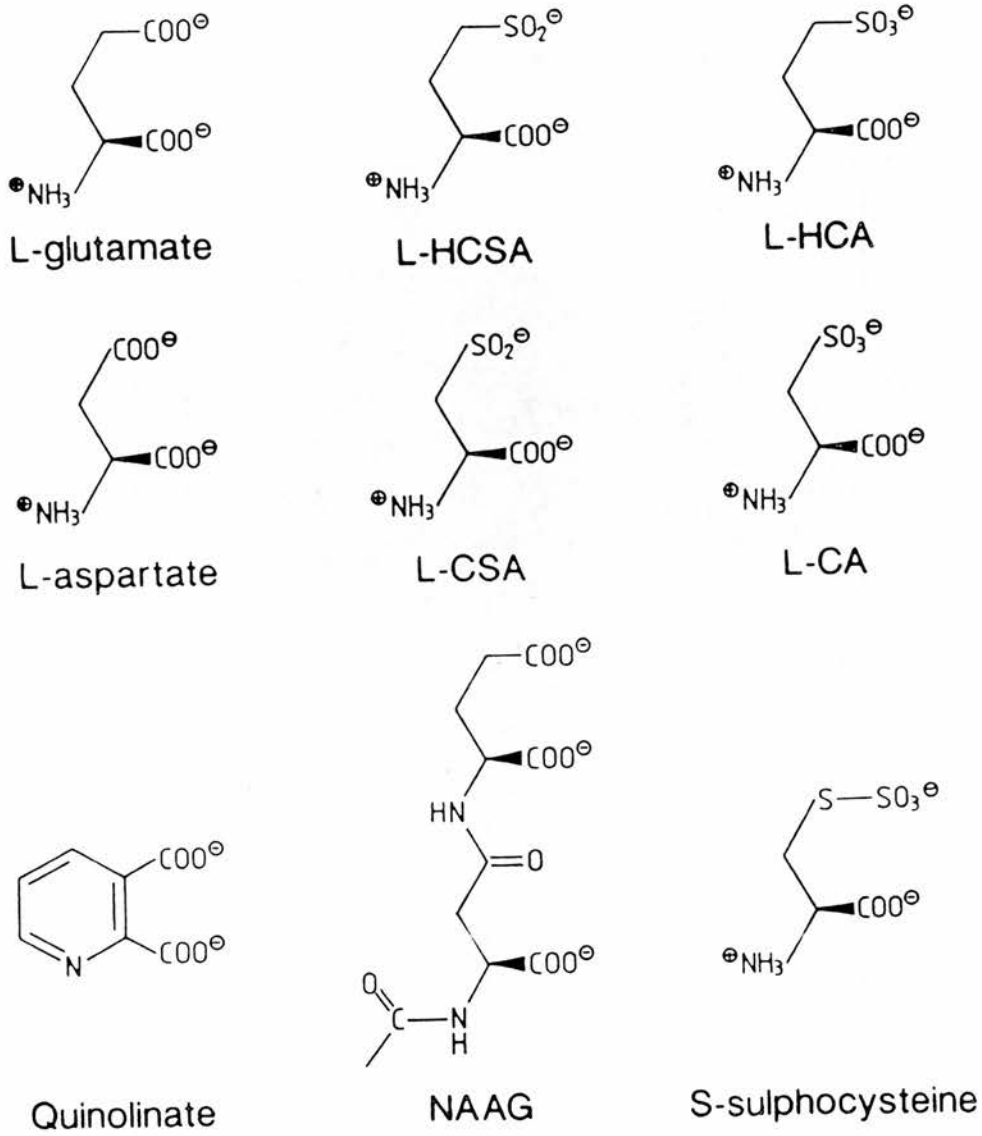

FIGURE 1 : Chemical structures of endogenous EAA agonists
Abbreviations: L-HCSA, L-homocysteine sulphinic acid; L-HCA, L-homocysteic acid; L-CSA, L-cysteine sulphinic acid; L-CA, L-cysteic acid; NAAG, N-acetyl-L-aspartyl-L-glutamate

the dorsal and ventral roots of the spinal cord (Fonnum, 1984; Graham *et al*, 1967). It plays an integral role in metabolic processes namely deamination, transamination and amidation reactions which are involved in the detoxification of ammonia in the CNS (Weil-Malherbe, 1950). This ubiquitous distribution and integral role in cell metabolism makes it very difficult to separate the metabolic pool of L-glutamate from the neurotransmitter pool. L-Glutamate is also a precursor of the inhibitory neurotransmitter, GABA (Roberts and Frankel, 1950).

The synthesis of neurotransmitter L-glutamate has been shown to be extremely complex (Fonnum, 1984). Glucose, acetate, glutamine, 2-oxyglutarate and ornithine have all been proposed as putative precursors (Fig.2), (Potashner, 1978; Reijnierse *et al*, 1975; Hamberger *et al*, 1979a,b; Fonnum, 1984; Yoneda *et al*, 1982).

Glutamine is probably the major precursor candidate for neurotransmitter L-glutamate synthesis (Fig.2B; Shank and Campbell, 1983). Phosphate activated glutaminase, which converts glutamine to L-glutamate, has been demonstrated in nerve terminals (Kvamme, 1983; Ward *et al*, 1982). Double labelling release studies resulted in four times more L-glutamate derived from glutamine being released as that derived from glucose (Hamberger *et al*, 1979a,b; Bradford *et al*, 1978). A major proportion of the glutamine is thought to originate from L-glutamate breakdown in glial cells. Preloading synaptosomes with glutamine has been shown to increase L-glutamate release indicating that newly acquired glutamine is readily converted into a releasable pool of L-glutamate (Johansen *et al*, 1987).

Brain slice and synaptosomal membrane preparations are commonly used to demonstrate both neurotransmitter release and re-uptake systems. Using a variety of depolarising stimuli, Ca^{2+}-dependent release of radiolabelled L-glutamate has been demonstrated (Potashner, 1978; Nadler *et al*, 1978; Toggenburger *et al*, 1982). Release of endogenous L-glutamate has been measured both in vivo (Abdul-Ghani *et al*, 1979) and in vitro (Malthe-Sorenssen *et al*, 1980; Collins, 1980). Depolarisation of glial preparations also results in release of L-glutamate but in a Ca^{2+}-independent manner (Duce and Keen, 1983). Release studies have also been used in an effort to

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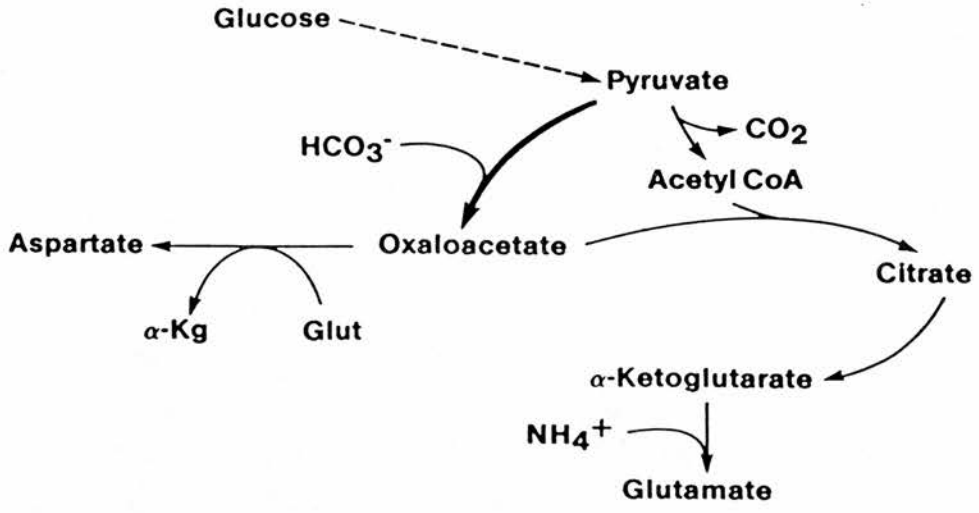

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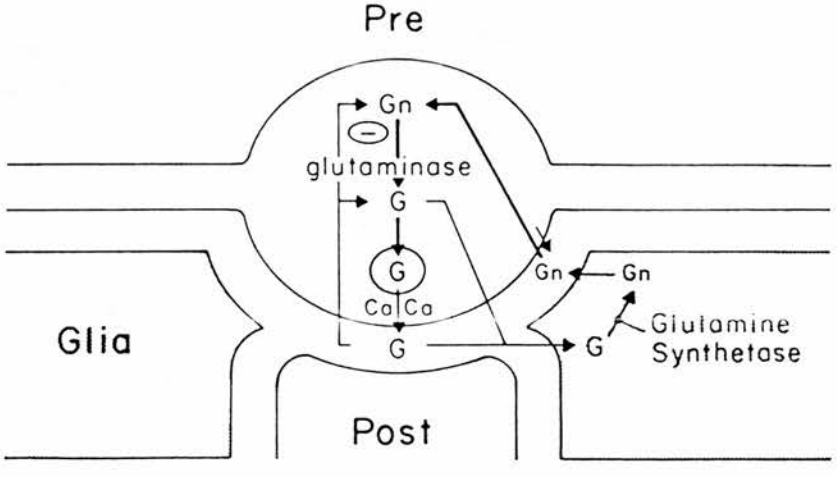


FIGURE 2 ; Synthetic and metabolic pathways of neurotransmitter L-glutamate

A. One of the many proposed synthetic pathways of neurotransmitter L-glutamate, in this case the substrate is glucose. The relationship with the synthesis of L-aspartate is clearly shown.

B. Diagrammatic representation of the L-glutamate-glutamine cycle in L-glutamate releasing neurons and associated glial cells. Abbreviations: G, L-glutamate; Gn, glutamine; Ca, calcium ions.

distinguish between the neurotransmitter and metabolic pools of L-glutamate. Decortication of rats resulted in the selective loss of L-glutamate release from the striatum, this is presumed to be the transmitter pool. The large reduction following kainate lesioning of the striata probably reflects the metabolic pool (Butcher and Hamberger, 1987).

Using synaptosomal preparations L-glutamate uptake into neurones was shown to be Na^+-dependent, requiring two Na^+ ions per molecule of L-glutamate (Bennet *et al*, 1973; Stallcup, 1979). Na^+-Dependent glial cell uptake is also an important process for removing L-glutamate from the synaptic cleft (Hertz, 1975) (Fig.2B). Decreased L-glutamate uptake after lesioning of presumed glutamatergic pathways has been demonstrated in the nucleus accumbens and caudate putamen (Walaas, 1981) as well as other subcortical areas (Young *et al*, 1981). The neuronal and the glial cell uptake system are both selective for L-glutamate and D and L-aspartate, with no other amino acids being transported to any great extent (Davies and Johnston, 1976). Glutamate-induced excitation is therefore prolonged in the presence of uptake inhibitors (Johnston *et al*, 1980).

Finally, L-glutamate has been demonstrated to interact with specific receptors in the mammalian CNS. Exogenous application of L-glutamate mimics the actions of endogenous L-glutamate (Curtis and Watkins, 1960). The identification of antagonists of L-glutamate induced excitations have helped in the definition of specific EAA receptors (Watkins, 1986). Although three L-glutamate receptors were initially classified (Watkins and Evans, 1981), five receptor subtypes (Table 2) have been identified with the possibility of further subclasses (see Watkins, 1989; Monaghan *et al*, 1988).

L-Aspartate

L-Aspartate (Fig.1) certainly cannot be discounted as a central excitatory neurotransmitter although its effects cannot be easily separated from those of L-glutamate, because they are metabolically related through a transamination reaction

(Fonnum, 1984). L-Aspartate may be synthesised as a biproduct of a L-glutamate synthetic pathway (Fig.2). Both amino acids are substrates for the same uptake carrier (Davies and Johnston, 1976). In vivo they are both released upon stimulation and interpretation of uptake and release studies is therefore very difficult (Watkins and Evans, 1981). Most of what has already been discussed in the preceding section on L-glutamate also applies equally to L-aspartate. Uptake studies show similar ratios between L-aspartate and L-glutamate throughout the brain (McGeer *et al*, 1977).

Differences have, however, been clearly observed between the binding specificities of [^{3}H]L-aspartate and [^{3}H]L-glutamate to synaptic membrane preparations (Foster *et al*, 1981a). Electrophysiological studies have also demonstrated differences in responses to L-aspartate and L-glutamate (Duggan, 1974; Davies and Watkins, 1973).

Other EAA transmitter candidates

Curtis and Watkins (1963) originally demonstrated the potency of sulphur containing amino acids as neuronal excitants (Fig.1). Most evidence points to L-homocysteate (L-HCA) as having an excitatory neurotransmitter role, since it is twelve times more potent than L-glutamate as an excitant of central neurones and has a selective action at the NMDA receptor (Mewett *et al*, 1983).

L-HCA release from brain slices was higher than for other sulphur containing homologues with L-HCA-like immunoreactivity and L-HCA release both being highest in brain areas containing a high density of NMDA receptors (Do *et al*, 1986b). (±)-ß-p-Chlorphenylglutamate an amino acid uptake blocker (Davies *et al*, 1985) prolonged the depolarisation affect of L-HCA but did not effect the responses of other amino acids on brain slices, indicating a specific uptake system for L-HCA (Zeise *et al*, 1988). The NMDA antagonists 2-amino-5-phosphonopentanoate (AP5) and 2-amino-7-phosphonoheptanoate (AP7) abolished the NMDA response, markedly reduced the L-HCA response, but had no effect on the L-glutamate induced depolarisations (Do *et al*, 1986a; Knopfel *et al*, 1987). L-HCA has similar stimulatory actions to L-glutamate on

[^{3}H]dizocilpine binding, an effect blocked by CPP and potentiated by glycine (Schwarz *et al*, 1990). This is an indication that the site of action is the L-glutamate neurotransmitter site. The excitotoxic effect of L-HCA has a similar pharmacological profile to that of NMDA but not kainate (KA) (Olney *et al*, 1987). No definitive evidence exists yet as to the synthesis of L-HCA within the CNS but Griffiths *et al* (1992) suggest that it may be analogous to the synthesis of L-cysteic acid. Electrophysiological and biochemical evidence would certainly support the view that if L-HCA and other sulphur containing amino acids are central neurotransmitters they act specifically on the NMDA receptor. They may be responsible for the neurotoxic effects caused by activation of the NMDA receptor (Olney *et al*, 1990).

N-Acetyl-L-aspartyl-L-glutamate (NAAG) is a dipeptide containing both L-glutamate and L-aspartate within its structure (Fig.1). First identified around twenty five years ago (Curatola *et al*, 1965), Zaczek *et al* (1983) have proposed it as a central neurotransmitter candidate. NAAG is now well characterised as a potential excitatory neurotransmitter. It has a defined regional distribution (Koller *et al*, 1984) and NAAG-like immunoreactivity (NAAG-LI) exhibits a high degree of localisation within glutamatergic neurones (Coyle *et al*, 1989), as well as co-localisation in groups of cholinergic, serotonergic and noradrenergic neurones (Forloni *et al*, 1987; Guarda *et al*, 1988). This may be suggestive of NAAG having a role as a neuromodulator rather than a neurotransmitter. The actions of NAAG are terminated by enzymatic hydrolyses with high affinity reuptake of breakdown products (Robinson *et al*, 1987; Coyle *et al*, 1989). NAAG has been found to depolarise central neurones whilst its breakdown product N-acetylaspartate (NAA) is inactive thereby suggesting excitatory activity is due only to the peptide itself (ffrench-Mullen *et al*, 1985). However, L-glutamate itself is also liberated (Coyle *et al*, 1989), and thus may be responsible for the neurotransmitter actions of NAAG. Electrophysiologically NAAG has been shown to have both NMDA and non-NMDA characteristics (ffrench-Mullen *et al*, 1985; Westbrook *et al*, 1986).

Quinolinic acid (Fig.1) is an endogenous tryptophan metabolite and is a potent excitant comparable with L-glutamate on single cortical neurones (Stone and Perkins, 1981). This excitatory activity is antagonised in parallel with NMDA by AP5, AP7 and HA-966 (3-amino-1-hydroxypyrrolidin-2-one) in vivo and in vitro (Perkins and Stone, 1983; 1985). Wolfensberger *et al* (1983) have identified low concentrations of endogenous quinolinate in both rat and human brain (<1μM). Distinct regional variations in quinolinate concentration have been measured as well as an increase in concentration with age (Moroni *et al*, 1984a,b). Unusually, quinolinate does not activate all NMDA-sensitive neurones, suggesting that it may be a selective agonist at a specific subtype of NMDA receptor (Perkins and Stone, 1983). Herrling *et al* (1983) provided further evidence for NMDA selectivity by observing that quinolinate mimicked NMDA but not quisqualate responses. Quinolinate is not a substrate for the L-glutamate uptake system and there is no evidence for uptake of [^{3}H]-quinolinate into synaptosomes or brain slices (Balcar and Johnston, 1972; Foster and Fagg, 1984; Collins *et al*, 1985). The main argument against quinolinate being a neurotransmitter is probably the lack of an uptake system to terminate its actions. Continual quinolinate stimulation could lead to receptor desensitisation and potentially to neurotoxicity mediated via the NMDA receptor, and so may therefore have greater pathological than physiological importance (Stone and Connick, 1985). Quinolinate has indeed been implicated as a mediator of neurodegeneration in Huntington's chorea (Beal *et al*, 1986), as well as in HIV-1 infected patients where increases in CSF quinolinate have been detected (Heyes, 1990).

Identification of EAA receptors

The neurotransmitter candidates discussed in the preceding section elicit their action by interacting with one or more of the identified postsynaptic EAA receptors including the NMDA receptor. McLennan *et al* (1968) demonstrated electrophysiologically a regional difference in the CNS in the potency of L-glutamate. This was confirmed by Duggan (1974) and McCulloch *et al* (1974) who found that

neurones in the dorsal horn of the spinal cord were more sensitive to L-glutamate than L-aspartate and that NMDA and KA were acting on different receptors. Davies and Watkins (1973) went on to demonstrate that HA-966 was more effective at blocking L-aspartate elicited depolarisations than those of L-glutamate. This led to proposals of more than one EAA receptor. The later observation that Mg^{2+} had a more profound effect on NMDA and L-aspartate induced excitations than those elicited by kainate (KA), quisqualate (QA) and L-glutamate (Evans *et al*, 1977; Ault *et al*, 1980) proved to be of great importance in the differentiation of EAA receptors.

A major step forward came when Monaghan *et al* (1983) using [^{3}H]L-glutamate demonstrated the existence of four separate EAA receptors autoradiographically, with high affinities for NMDA, AMPA (α-amino-2-hydroxy-5-methyl-4-isoxazole propionic acid), L-AP4 (2-amino-4-phosphonbutanoate) and KA. These studies compared well with known electrophysiological and neurotoxicity data.

[^{14}C]L-glutamate was the first radioligand used to study the binding of L-glutamate to synaptic membranes, (Roberts, 1974; Michaelis *et al*, 1974). These two independent studies found that the binding was saturable, of high affinity and was displaced by other neuroexcitatory amino acids. This was the first indication of a receptor interaction. L-glutamate binding studies were hampered until [^{3}H]L-glutamate of a higher specific activity became available and receptor selective drugs were introduced. This led to the demonstration that detectable binding was specific for the CNS and not the periphery (Biziere *et al*, 1980; Foster and Roberts, 1978). Not only was binding specific for the CNS but it was specifically located in the synaptic junction (Foster and Roberts, 1978; Foster *et al*, 1981b), indicating a synaptic role for L-glutamate.

A wide range of values for binding site affinity (K_d) and binding site density (B_{max}) have been reported for [^{3}H]L-glutamate binding depending on the membrane preparation used, the ions present in the assay and other alterations in assay conditions

(see Foster and Fagg, 1984). Foster and Fagg (1984) have divided [^{3}H]L-glutamate binding up into high, medium and low affinity binding sites, subdivided by further ionic specificity.

The advent of specific NMDA antagonists radiolabelled to a high specific activity has enabled research on the NMDA receptor to proceed most rapidly. The ligands used to identify the non-NMDA receptors apart from [^{3}H]CNQX (Nielsen *et al*, 1990) however are still radiolabelled selective agonists (Table 1). The five EAA receptors classified pharmacologically are shown in Table 2.

Over the past few years there have been rapid advances made in the cloning and purification of L-glutamate receptor subunits. The G-protein linked metabotropic receptor has been demonstrated to have similarities to other known G-protein coupled receptors which stimulate phosphoinositide metabolism in that it possesses a large amino-terminal extracellular domain and the putative 7 transmembrane segments (Masu *et al*, 1991).

The ionotropic EAA receptors have also been cloned and in common with other ligand-gated ion channels (Betz, 1990) they have four putative transmembrane segments arranged in a similar conformation. However many structural features also differ (Barnes and Henley, 1992). This may be suggestive of a superfamily of ionotropic EAA receptors (Mayer, 1991). However, certain features do not appear to be conserved even within the EAA receptor subgroup. There is certainly still much to be investigated regarding the molecular structure of EAA receptors, which reveals a wide diversity of EAA receptor subtypes and will help in the understanding of their molecular mechanisms.

Physiological and pathological roles of EAA receptors

The NMDA receptor has been identified as playing a role in normal synaptic transmission (Davies, 1989) as well as in the phenomenon of long term potentiation (LTP; Collingridge *et al*, 1983). LTP is thought to be a good cellular correlate for learning and memory. Morris *et al* (1986) have demonstrated that the NMDA

TABLE 1 RADIOLIGANDS COMMONLY EMPLOYED TO INVESTIGATE THE EXCITATORY AMINO ACID RECEPTORS

Receptor	NMDA	AMPA	KAINATE	L-AP4	METABOTROPIC
Ligands	[^{3}H]-L-glutamate	[^{3}H]-L-glutamate	[^{3}H]-L-glutamate	[^{3}H]-L-glutamate	[^{3}H]-L-glutamate
	[^{3}H]-D-AP5	[^{3}H]-AMPA	[^{3}H]-kainate	[^{3}H]-L-AP4	
	[^{3}H]-CPP	[^{3}H]CNQX			
	[^{3}H]-CGS19755				
	[^{3}H]-Dizocilpine[1]				
	[^{3}H]-PCP/TCP[1]				
	[^{3}H]-glycine[2]				
	[^{3}H]-CGP39653				
	[^{3}H]-5,7-dichlorokynurenate[2]				
	[^{3}H]-L-689,560[2]				

[1] Binds to a site within the associated ion channel.
[2] Binds to the associated strychnine insensitive glycine modulatory site.

Abbreviations: AP5, 2-amino-5-phosphonopentanoate; CPP, 3-((-)-2-carboxypiperazin-4-yl)propyl-1-phosphonate; CGS19755, 1-(cis-2-carboxypiperidine-4-yl)methyl-1-phosphonate; PCP, phencyclidine; TCP, N-[1-thienyl]-cyclohexyl-3,4-piperidine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; AP4, 2-amino-4-phosphonobutanoate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

TABLE 2 CLASSIFICATION OF THE EXCITATORY AMINO ACID RECEPTORS

RECEPTOR	NMDA	AMPA*	KAINATE	L-AP4	METABOTROPIC
Specific agonists	NMDA	AMPA	Kainate	L-AP4	ACPD
Competitive antagonists	D-AP5 D-AP7 D-CPP CGS19755 CGP37849	CNQX DNQX NBQX GAMS	CNQX DNQX MNQX GAMS		
Non-competitive antagonists 1. Exogenous	Ketamine PCP/TCP Dizocilpine SKF 10,047 argiotoxin 636				
2. Endogenous	Mg2+				
Allosteric Modulators 1. Glycine site agonists antagonists	Glycine D-serine HA-966 Kynurenate 7-chloro-kynurenate cycloleucine ACC MNQX				
2. Miscellaneous Modulators	Ifenprodil Zinc SL 82.0715				

* The AMPA receptor was previously named the quisqualate receptor.

Abbreviations: NMDA, N-methyl-D-aspartate; AP5, 2-amino-5-phosphonopentanoate; AP7, 2-amino-7-phosphonoheptanoate; CPP, 3-((-)-2-carboxypiperazin-4-yl)propyl-1-phosphonate; CGS 19755. 1 (cis-2-carboxypiperidine-4-yl)methyl-1-phosphonate; PCP, phencyclidine; TCP, N-[1-thienyl]-cyclohexyl-3,4-piperidine; HA-966, 3-amino-1-hydroxypyrrolidin-2-one; ACC, 1-aminocyclopentanecarboxylate; MNQX, 5,7-dintroquinoxaline-2,3-dione; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NBQX,6-nitroquinoxaline-2,3-dione; GAMS, δ-D-glutamyl aminomethyl sulphonate; AP4, 2-amino-4-phosphonobutanoate; ACPD, trans-l-amino-cyclopentyl-1,3-dicarboxylate.

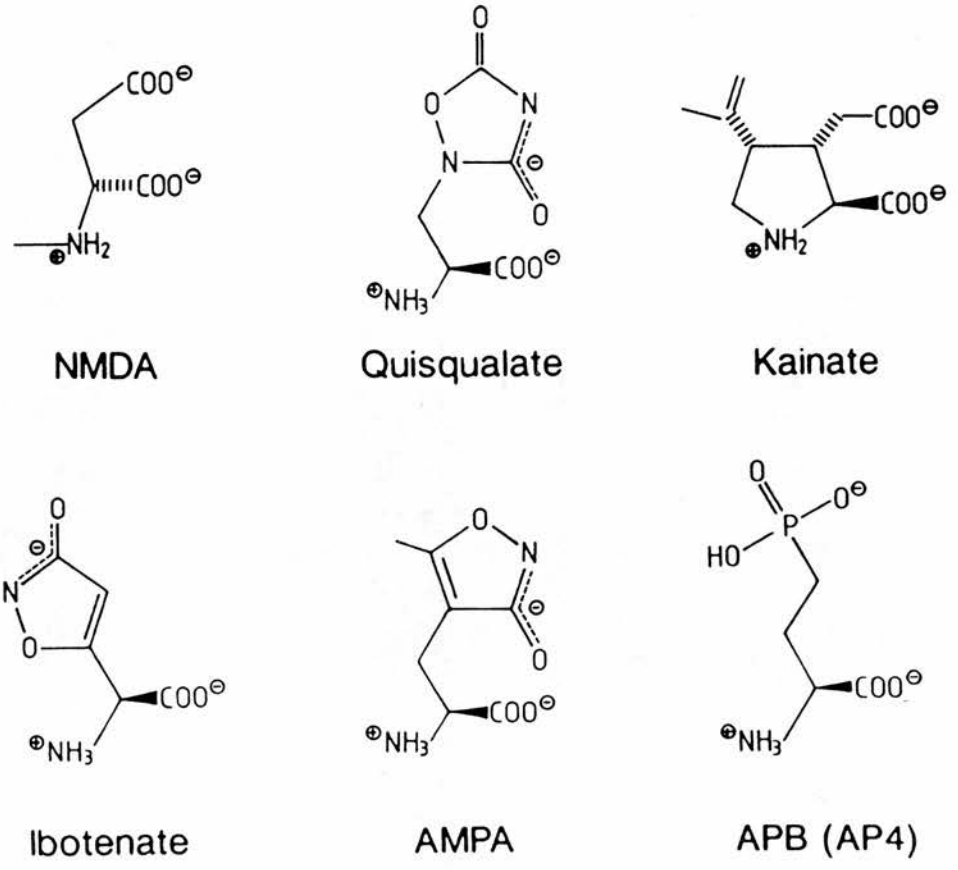


FIGURE 3: Chemical structures of exogenous EAA agonists
Abbreviations: NMDA, N-methyl-D-aspartate; AMPA,x-amino-3-hydroxy-5-methyl-4-isoxazole propionate.

antagonist D-AP5 impairs learning and memory. The NMDA receptor is also involved in epileptiform burst discharges and has been implicated in the development of neurones in the CNS (Ben-Ari and Represa, 1989). It is thought that inappropriate activation of NMDA receptors may be responsible for neurodegeneration in a number of identified pathological conditions including Alzheimer's disease, Huntington's chorea (HC) and excitotoxic neural damage resulting from hypoxic ischaemic insults (Bullock *et al*, 1990; Bridges *et al*, 1988; Woodruff *et al*, 1988). A more extensive list of clinical conditions in which the NMDA receptor is implicated is given in Herrling (1989). The non-NMDA receptors have also has roles assigned to them. KA and AMPA receptors are also important in LTP and in fast synaptic transmission (Collingridge and Lester, 1989). The KA/AMPA antagonist 2,3-dihyroxy-6-nitro-7-sulfamyl-benzo (F) quinoxaline, (NBQX) has been demonstrated to act as a neuroprotectant in cerebral ischaemia (Sheardown *et al*, 1990), thus these receptors are important in mediating neurotoxic cell damage. The metabotropic receptor has been implicated in neurodegenerative disease, and in neuronal developmental plasticity (Cha *et al*, 1990; Nicoletti *et al*, 1988). Recent evidence also proposed a role for this receptor in synaptic transmission (Miller, 1991a).

1.3 NON-NMDA EAA RECEPTORS

The four non-NMDA receptors are poorly characterised compared with the NMDA receptor. This is due to the lack of compounds acting selectively at any one of the non-NMDA sites. Agonists are still used in preference to the few non-selective antagonists available, to investigate these receptors (Fig.3). Two of these receptors are linked directly to ion channels (KA and AMPA receptors). The physiological mechanism of the L-AP4 receptor is as yet poorly understood. KA and QA (Fig.3) are both plant extracts and potent excitants of central neurons acting at EAA receptors (Takemoto, 1978; Shinozaki *et al*, 1974; Biscoe *et al*, 1976). AMPA (Fig.3) is more selective than QA at the ionophore linked QA receptor, thus the ionotropic QA receptor is now termed the AMPA receptor (Krogsgaard-Larsen *et al*, 1980; Sugiyama *et al*,

1988). Due to the lack of selective antagonists it has been difficult to separate KA and AMPA responses. Both receptors are linked to low conductance ion channels which conduct Na^+ and K^+ in a voltage independent manner (Ascher and Nowak, 1988b; Crunelli *et al*, 1984; MacDonald *et al*, 1982). Radioligand binding studies have demonstrated differences in receptor distribution using [^{3}H]AMPA, [^{3}H]CNQX and [^{3}H]KA (Nielsen *et al*, 1990; Slevin and Coyle, 1981). The distribution of AMPA receptors resembles that of NMDA receptors (high in hippocampal CA1 area, outer cortical layers and molecular layer of the cerebellum) as opposed to KA receptor distribution (highest in the CA3 region of the hippocampus, deep cortical layers, striatum and granule cell layer of the cerebellum) (Monaghan *et al*, 1983; 1985; Cha *et al*, 1988). Although there are clear differences, there is a degree of response overlap which has been explained as both responses being mediated through the same receptor-channel complex with differences in desensitisation or conductance states accounting for the varied responses (see Collingridge and Lester, 1989; Nicoll *et al*, 1990). Cross-desensitisation studies may help to understand the situation fully. This doubt about the KA/AMPA receptor(s) and ion channel(s) is still apparent even though a functional KA receptor which is insensitive to QA and a family of AMPA-selective receptors have been cloned (Hollman *et al*, 1989; Keinanen *et al*, 1990).

The quinoxalinediones are the most effective KA/AMPA antagonists available to date (Honore *et al*, 1988; Blake *et al*, 1988; Drejer and Honore, 1988), although they are also reported to interact with the NMDA glycine site (Birch *et al*, 1988a,b). Electrophysiological studies have shown that 6-cyano-7-nitroquinoxaline-2,3-dione (Fig.7; CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) depress AMPA responses to a greater, lesser or similar extent than to KA-responses (Neuman *et al*, 1988; Andreasen *et al*, 1989; Sheardown *et al*, 1988). It has again been suggested that this mixed effect may be due to AMPA and KA acting at the same receptor. Selective antagonists are required to investigate this possibility. They would also aid investigation into the roles of the individual receptors in fast excitatory synaptic transmission. NBQX has been reported to be more selective for AMPA than KA

responses, and it has been recently suggested that argiotoxin may be used to separate the responses (Lodge *et al*, 1990). Both receptors can mediate neurotoxic cell damage therefore selective antagonists have a potential therapeutic use (Drejer and Honore, 1988).

Activation of the metabotropic receptor, which is linked to a G-protein, by L-glutamate leads to the metabolism of inositol phosphate (IP). This was initially demonstrated in Xenopus oocytes (Gundersen *et al*, 1984; Parker and Miledi, 1987; Sugiyama *et al*, 1987). This effect was mimicked by L-glutamate and QA but not by NMDA, KA or L-AP4 (Sugiyama *et al*, 1988). A similar response was later identified in mammalian brain (Palmer *et al*, 1988). In cortical cell cultures this response was also resistant to antagonism by CNQX (Patel *et al*, 1990). Thus a QA response had been identified which was not mediated via the conventional QA/AMPA receptor. *Trans*-l-amino-cyclopentyl-1,3-dicarboxylate (ACPD) is the most selective agonist at this receptor, evoking the rapid formation of IP_1, IP_2, and IP_3 and IP_4 in a similar way to QA (Monaghan *et al*, 1989). AMPA insensitive, QA sensitive [^{3}H]L-glutamate binding has been used to identify presumed metabotropic receptors. Highest densities are observed in hippocampus, cortex and striatum. The receptor has been cloned, revealing a family of metabotropic L-glutamate receptors which are linked to other intracellular biochemical pathways in addition to IP metabolism and have differential sensitivity to ACPD (Masu *et al*, 1991; Tanabe *et al*, 1992).

The L-AP4 receptor is named after the selective compound active at this site. L-AP4 (Fig.3) unlike AP5 and AP7 is relatively inactive at the NMDA receptor (Olverman and Watkins, 1989). No other compounds selective for this site have been identified, thus precluding further investigations. L-AP4 does block synaptic transmission to varying degrees in the hippocampus and the spinal cord with the response being sensitive to broad spectrum EAA antagonists indicating a presumptive receptor interaction (Evans *et al*, 1982; Koerner and Cotman, 1981). More recent electrophysiological evidence points to the receptor being presynaptic with a role in blocking neurotransmitter release though how this is achieved is unclear (Cotman *et al*,

1986. Binding studies using [^{3}H]L-glutamate and [^{3}H]L-AP4 indicate a post synaptic interaction at a site where NMDA, AP5 and AP7 have no effect (Fagg *et al*, 1982; Butcher *et al*, 1983; Monaghan *et al*, 1983). The studies using [^{3}H]L-glutamate were performed in the presence of Cl^- and it is now proposed that the binding is to a Cl^- dependent L-glutamate transporter (Pin *et al*, 1984; Fagg and Lanthorn, 1985). Clearly more work is necessary to fully understand the complexity of the response to L-AP4 and its physiological relevance.

1.4 THE NMDA RECEPTOR

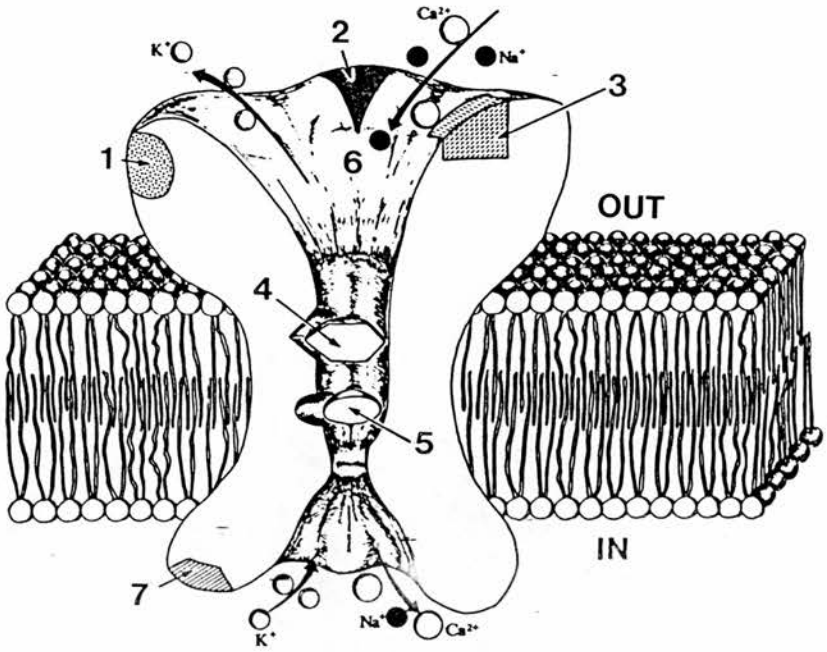
The NMDA receptor (Fig.4) is by far the best characterised EAA receptor in the mammalian CNS. This is mainly due to the work of Watkins and colleagues who, synthesised potent antagonists for EAA receptors, the NMDA receptor in particular. Verification of the actions of the novel compounds was confirmed electrophysiologically as well as by radioligand binding studies.

Although initial studies focussed on the neurotransmitter site of the NMDA receptor it was found in due course to be a complex structure with multiple sites available for pharmacological interaction (Fig.4). The main sites identified to date are as follows:

1. Neurotransmitter recognition site.
2. Allosteric glycine modulatory site.
3. Mg^{2+} binding site.
4. PCP/Dizocilpine binding site.
5. Zn^{2+}/Tricyclic antidepressant (TCA) site.
6. Polyamine site.

Pharmacological characterization

NMDA (Fig.3) is a synthetic amino acid first synthesised around thirty years ago (Watkins, 1962). Until the discovery of KA and QA it was the most potent neuroexcitant known because it is not a substrate for the selective L-glutamate uptake

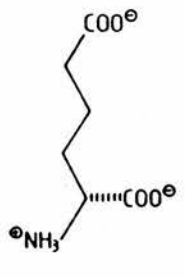


1. Neurotransmitter binding site.
2. Allosteric glycine modulatory site.
3. Zn^{2+}/TCA site.
4. PCP/Dizocilpine binding site.
5. Mg^{2+} binding site.
6. NMDA ion channel.
7. Proposed site of action of the polyamines.

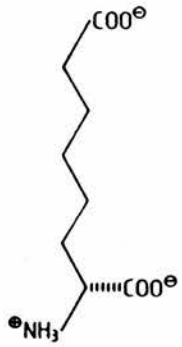
FIGURE 4: Schematic representation of the NMDA receptor complex
Identified sites are clearly illustrated. Abbreviations: TCA, tricyclic antidepressant; PCP, phencyclidine.

Modified from Wong, E.H.F. and Kemp, J.A. (1991) Annual Review of Pharmacology and Toxicology, 31, 401-425.

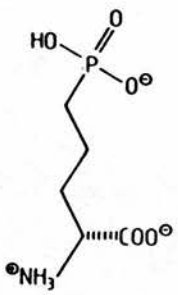
FIGURE 5: Chemical structures of competitive NMDA antagonists
Abbreviations: DAA, D-a-aminoadipate; DAS, D-a-aminosuberate; AP5, 2-amino-5-phosphonopentanoate; AP7, 2-amino-7-phosphonoheptanoate; CGS, 1-(cis-2-carboxypiperidine-4-yl)methyl-1-phosohonate(CGS 19755); CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate; CPP-ene,3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate; CGP 37849, 2-amino-4-methyl-5-phosphono-3-pentenoate; CGP 39551, the carboxyethylester of CGP 37849.



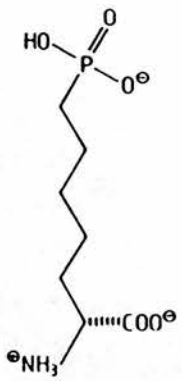
DAA



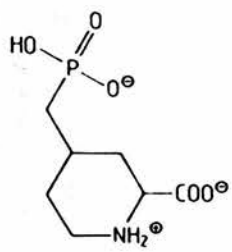
DAS



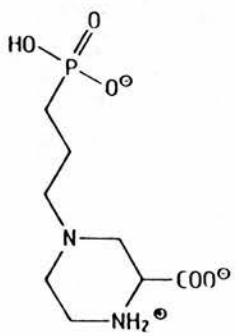
APV (AP5)



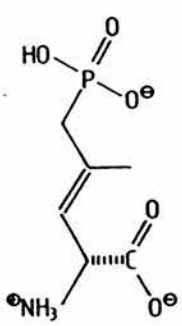
APH (AP7)



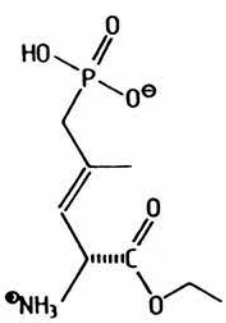
CGS



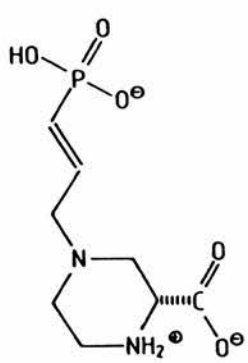
CPP



CGP 37849



CGP 39551



CPP-ene

system (Balcar and Johnston, 1972). Apart from NMDA other exogenous agonists at this receptor include ibotenate (Fig.3) and *trans* 2,3 piperadine dicarboxylate (*trans*-2,3-PDA) (Curtis *et al*, 1979; Watkins and Olverman, 1988). Endogenous agonists at the NMDA receptor have already been discussed (Section 1.2).

HA-996 and Mg^{2+} were proposed as the first selective NMDA antagonists (see Watkins, 1986). These were superceded by more selective NMDA antagonists of low potency which were long chain analogues of L-glutamate. These included D-α-aminoadipate (D-AA) and D-α-aminosuberate (D-AS) (Fig.5; Davies and Watkins, 1973; Evans *et al*, 1978, 1977; Collingridge and Davies, 1979; Watkins and Olverman, 1988). These compounds were termed first generation NMDA antagonists and helped to provide much of the evidence for the existence of NMDA and non-NMDA receptors.

Second generation NMDA antagonists include the compounds D-AP5 and D-AP7. Both are members of the α-amino-ω-phosphonodicarboxylate homologous series of compounds, being analogues of D-AA and D-AS respectively (Fig.5; Davies *et al*, 1981; Watkins and Evans, 1981). The homologues, D-AP4, D-AP6 and D-AP8 were considerably less active as antagonists at the NMDA receptor (Evans *et al*, 1982; Watkins and Olverman, 1988). The D or (-) isomer of AP5 was reported to be most active (Davies and Watkins, 1982; Watkins and Olverman, 1987). It has been the compound of choice for much of the functional characterisation of the NMDA receptor having a higher affinity than D-AP7 for inhibition of [^{3}H]AP5 binding (Watkins and Olverman, 1988). K_i values for inhibition of L-[^{3}H]L-glutamate binding showed a similar pattern (Fagg and Baud, 1988). D-AP5 has no effect on responses elicited by other central neurotransmitters (Collingridge *et al*, 1983; Childs *et al*, 1988). It is also without effect on responses elicited by KA, QA and AMPA (Davies *et al*, 1981). Schild plot analysis of electrophysiological data revealed that D-AP5 is a competitive antagonist at the NMDA receptor (Evans *et al*, 1982). Although D-AP5 is a very useful experimental tool, potentially it has a very limited use as a therapeutic agent

because of its relative inability to cross the blood brain barrier (BBB) (Meldrum *et al*, 1985). This led to a third generation of competitive antagonists which are generally more lipophilic than AP5 and AP7.

The first effective compound was a conformationally restricted analogue of D-AP7, 3-((-)-2-carboxypiperazine-4-yl)propyl-1-phosphonate (CPP) (Davies *et al*, 1986; Murphy *et al*, 1987; Lehmann *et al*, 1987). CPP (Fig.5) is three times more potent than D-AP5 as an antagonist at NMDA receptors as shown by inhibition of [^{3}H]D-AP5 and [^{3}H]CPP binding (K_i values of 0.48μM and 0.35μM respectively compared with 1.4μM and 1.2μM respectively for D-AP5; see Watkins and Olverman, 1988). The D-isomer of CPP has since been reported to be most active (Herrling *et al*, 1989). Many more competitive NMDA antagonists are now available, all analogues of AP5 or AP7 (Fig.5). These include CGS 19755 (Lehmann *et al*, 1988; Murphy *et al*, 1988), CPPene (Herrling *et al*, 1989; Aebischer *et al*, 1989), CGP 39551, CGP 37849 and CGP 40116 (Schmutz *et al*, 1989; Fagg *et al*, 1990). These compounds have K_i values for the inhibition of [^{3}H]CPP binding of approximately 130nM, 40nM, 310nM, 35nM and 19nM respectively. Clearly CGP 40116, an unsaturated analogue of D-AP5 and the D isomer of CGP 37849 has the highest affinity for the NMDA neurotransmitter recognition site. Since it is orally active it may prove to be not only a useful pharmacologic tool, but also to be of benefit therapeutically.

Further characterisation

The NMDA receptor has been characterised as being a protein component of the postsynaptic membrane (Fagg *et al*, 1986). The binding protein has been identified as being 209KDa using [^{3}H]CPP and molecular target size analysis (Honore *et al*, 1987). This is further proof that it is a separate structure from the KA and AMPA binding sites which have different molecular weights (Honore and Nielson, 1985; Honore *et al*, 1986). The same group also identified the NMDA sensitive [^{3}H]L-glutamate binding protein as being 121KDa, much smaller than the CPP site but similar to the size of the [^{3}H]N-[1-thienyl]-cyclohexyl-3,4-piperidine ([^{3}H]TCP) (118 kDa) and [^{3}H]glycine

sites (115 kDa) (Honore *et al*, 1987). They suggest that an additional macromolecule may have to be associated with the agonist binding site in order to bind antagonists (Honore *et al*, 1989). This evidence has been used in support of the suggestion that the ω-terminal of AP5 and AP7 bind to a site distinct from that at which agonists bind although part of their binding sites must be common (Watkins and Olverman, 1988; Fagg and Baud, 1988). The ω-terminal of these compounds may therefore be responsible for determining agonist or antagonistic properties.

An alternative view is that the recognition site exists in more than one conformation. The binding pattern of NMDA sensitive [^{3}H]L-glutamate (agonist) shows clear differences to that of [^{3}H]CPP (antagonist) although they are supposedly thought to be binding to the same site on the receptor (Monaghan *et al*, 1988). Originally, using NMDA sensitive [^{3}H]L-glutamate binding, high densities of NMDA receptors were found within the hippocampus, the nucleus accumbens and with a defined regional distribution in the cerebral cortex (Monaghan *et al*, 1985). Areas of lower NMDA receptor density include the globus pallidus and entorrhinal cortex. Identified EAA using pathways such as the dentate gyrus to CA3 and the cerebellar granule cell to purkinje cell had no detectable NMDA receptors. This is in agreement with electrophysiological studies which have demonstrated the lack of activity of NMDA antagonists at these synapses (Perkel *et al*, 1990).

In contrast, using [^{3}H]CPP as a radioligand, a slightly different pattern of binding was observed. While high levels of binding were still seen in the hippocampus the cerebral cortex had a more uniform distribution of binding. Levels higher than seen with [^{3}H]L-glutamate were observed in the thalamus while lower levels were seen in the medial and lateral septum. They concluded that there are agonist and antagonist preferring sites and that they show regional variations in their ability to bind radiolabelled agonists and antagonists (Monaghan *et al*, 1988). From this study it could be proposed that the cortex has more antagonist preferring sites while the medial

septum has more agonist preferring sites. Glycine is thought to play a modulatory role modifying the agonist/antagonist preferring state (Johnston and Ascher, 1987; Monaghan *et al*, 1988).

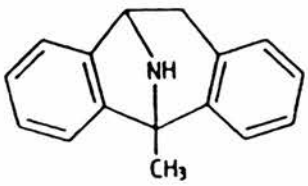
The NMDA associated ion channel and integral binding sites

The NMDA recognition site is linked and coupled to a cation conducting ion channel which spans the plasma membrane (Fig.4). L-glutamate has been shown to depolarise neurones by increasing Na^+ conductance (Nistri *et al*, 1985; Zanotto *et al*, 1983). Mg^{2+} had been shown to decrease NMDA-induced depolarisations in spinal cord (Evans *et al*, 1977; Davies and Watkins, 1977; Evans and Watkins, 1978). These surprising results were later explained by the discovery that the NMDA channel is blocked by extracellular Mg^{2+} in a voltage dependent manner (Nowak *et al*, 1984; Mayer *et al*, 1984). This was demonstrated in cultured neurons by the fact that the current-voltage (I/V) relationship for NMDA responses was non-ohmic, i.e. non linear. At potentials below -40mV a region of negative slope conductance was apparent. In the absence of Mg^{2+} the I/V relationship becomes linear again as it does when the membrane is depolarised (Ascher *et al*, 1988a; Mayer and Westbrook, 1984). Single channel studies suggest that Mg^{2+} binding site is located deep within the channel (Ascher *et al*, 1988). It seems that under normal physiological conditions of ~1mM Mg^{2+}, and at normal resting membrane potentials the NMDA receptors will probably be quiescent due to Mg^{2+} blocking the channel. However, Davies (1989) puts forward evidence suggesting that under normal physiological conditions NMDA receptor activation may be involved in synaptic transmission. This may be synapse or pathway selective.

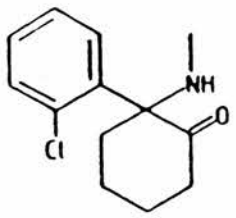
EAA receptor agonists have been shown to activate a number of single channel conductances although preferentially activating one conductance state (Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987). NMDA preferentially opened channels with the largest conductance states (40-50pS) which are the only conductances blocked by Mg^{2+}, an effect mimicked by Co^{2+} and Mn^{2+} (Ascher and Novak, 1988).

Unlike the AMPA and KA (but see Miller, 1991) ion channels, the NMDA channel in addition to conducting K^+ and Na^+ also conducts Ca^{2+} (MacDermott *et al*, 1986; Mayer *et al*, 1987). Ca^{2+} entry was studied using arsenazo III, a Ca^{2+} indicator dye. Extracellular Mg^{2+} blocked Ca^{2+} entry indicating that the Ca^{2+} originated extracellularly and was not being released from intracellular stores. Single channel recordings showed that only the 40-50pS channels were permeable to Ca^{2+} and the Ca^{2+} permeability of the NMDA channel is ten times higher than that of Na^+ (Jahr and Stevens, 1987). It has been postulated that one of the major functions of the NMDA receptor may be to increase intracellular Ca^{2+} concentrations at select locations.

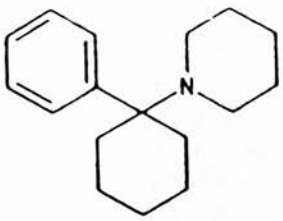
Apart from the endogenous Mg^{2+} block of the channel a number of exogenous agents act as channel blockers. The first identified blockers were the σ-opiates such as cyclazocine and SKF10,047 (a naloxone-insensitive effect) and the dissociative anaesthetics ketamine and phencyclidine (PCP; Fig.6) which selectively depressed responses to NMDA (Lodge *et al*, 1982; Anis *et al*, 1983; Berry *et al*, 1984). These channel blockers bind within the channel thereby decreasing channel open time (Macdonald *et al*, 1987). Dizocilpine (MK-801; Fig.6) is the most potent NMDA channel blocker found to date (Clineschmidt *et al*, 1982; Wong *et al*, 1986). These compounds are all non-competitive antagonists, i.e. use-dependent blockers, where the degree of antagonism can be enhanced by increasing the concentration of agonist present (Lodge *et al*, 1989). The block is greater when the cell is depolarised suggesting that the antagonist is trapped within the ion channel (Huettner and Bean 1988; MacDonald *et al*, 1987). Dizocilpine is thought to bind to a site distinct from that of Mg^{2+} although they may be allosterically related (Reynolds and Miller, 1988b). Further evidence for this is presented by Hori *et al* (1991). They demonstrated that Mg^{2+} had a biphasic effect on [^{3}H]TCP (Fig.6) binding (a stimulatory effect at low concentrations, EC_{50} = 11μM and an inhibitory effect at high concentrations, IC_{50} = 873μM). Similar effects were seen in the presence of L-glutamate and glycine. Hori *et al* (1991) go on to propose two Mg^{2+} sites on the NMDA receptor complex, the second site being of high affinity and coupled to a GTP binding site since guanine



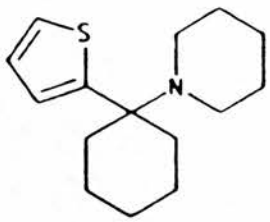
MK-801



Ketamine



PCP



TCP

FIGURE 6 ; Chemical structures of non-competitive NMDA antagonists
Abbreviations: MK-801, dizocilpine; PCP, phencyclidine; TCP, N-[1-thienyl]-cyclohexyl-3,4-piperidine

nucleotides blocked the Mg^{2+} stimulation. In the presence of agonist (L-glutamate) the binding of [^{3}H]dizocilpine, [^{3}H]TCP and [^{3}H]PCP is enhanced as shown by an increase in affinity for the binding site (Foster and Wong, 1987). Binding studies using [^{3}H]dizocilpine revealed that competitive antagonists had no effect on binding and likewise non-competitive antagonists have no effect on the binding of [^{3}H]competitive antagonists (Fagg and Baud, 1988; Wong *et al*, 1986). The regional distribution of [^{3}H]dizocilpine binding as measured autoradiographically is very similar to that seen for [^{3}H]AP5 (Bowery *et al*, 1988; Monaghan *et al*, 1986). This is a reflection of transmitter recognition site and associated ion channel co-localisation. Since PCP and dizocilpine bind with high affinity to their intra-channel site it could be that an endogenous compound (apart from Mg^{2+}) exists to modulate channel function (Zukin et al, 1987).

The non-competitive NMDA antagonists are much more lipophilic than competitive antagonists thereby having a higher bioavailability in the CNS after systemic administration (Kemp *et al*, 1986). The problem with these compounds as therapeutic agents is the incidence of side-effects as they interact with a number of other neurotransmitter systems linked to ion channels (Halliwell *et al*, 1989; Amador *et al*, 1991).

The Allosteric glycine modulatory site

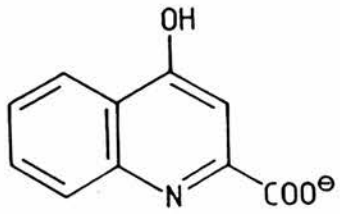
The amino acid glycine has traditionally been classed as an inhibitory neurotransmitter in the CNS, causing a hyperpolarisation when applied to central neurones. This response is abolished by strychnine. Glycine has recently been demonstrated to modulate responses elicited by the NMDA receptor by acting at a strychnine insensitive site (Johnson and Ascher, 1987).

NMDA responses recorded electrophysiologically from cultured neurones were smaller when a fast perfusion rate was used than when a slow perfusion rate was used. This suggested that perhaps an important endogenous modulatory substance was being washed away from the cell (Ascher and Johnston, 1989). Glycine was identified as this

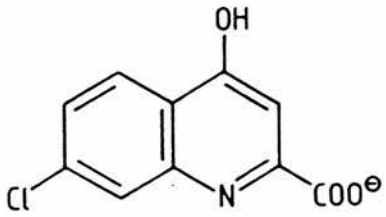
potentiating substance (Johnston and Ascher, 1987). Applied alone it has no effect but co-application with NMDA led to an increase in the amplitude of the NMDA response. Strychnine did not block the effect, therefore glycine was not acting at conventional inhibitory strychnine sensitive glycine receptors. This basic concept of glycine modulation resulted in the NMDA receptor complex being likened to the $GABA_A$ receptor which is modulated positively by the benzodiazepine class of drugs (Ascher *et al*, 1988b).

Strychnine insensitive [^{3}H]glycine binding has revealed a distinct population of sites in rat CNS. The distribution of sites matches that of [^{3}H]AP5 and therefore [^{3}H]dizocilpine but not of [^{3}H]strychnine indicating two populations of glycine receptors (Bristow *et al*, 1986; Monaghan *et al*, 1986). The binding of the ligands [^{3}H]dizocilpine, [^{3}H]TCP and [^{3}H]PCP to membrane preparations are all enhanced by addition of glycine (Ransom and Stec, 1988; Reynolds *et al*, 1987; Wong *et al*, 1987; Snell *et al*, 1988). This effect is manifest as a change in the affinity of [^{3}H]dizocilpine binding (Reynolds and Miller, 1988c). These results suggest a link between the glycine site and the NMDA channel.

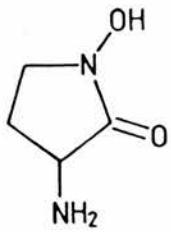
The discovery of this glycine site coupled to the NMDA receptor has aided in the understanding of the actions of kynurenate and HA-966 (Fig.7) on NMDA elicited responses. HA-966 has been identified as a glycine site antagonist on rat cortical wedge preparations (Fletcher and Lodge, 1988; Fletcher *et al*, 1989; Foster and Kemp, 1989). This effect can be reversed by addition of glycine. The effects of kynurenate are still "broad spectrum" at high concentrations but at lower concentrations it is a reasonably selective antagonist for the NMDA glycine site (Kessler *et al*, 1989). The 7-chloro derivative of kynurenate, 7-chlorokyunerate (7-Clkyn; Fig.7) and indole-2-carboxylic acid are also potent and selective antagonists at the glycine site (Kemp *et al*, 1988; Huetnner *et al*, 1989). The quinoxalinediones, CNQX and DNQX (Fig.7) should also be mentioned here. Apart from their activity at AMPA/KA receptors they also antagonise NMDA responses non-competitively through an action at the glycine site (Honore *et al*, 1988; Birch *et al*, 1988a; Lester *et al*, 1989). The newest antagonist



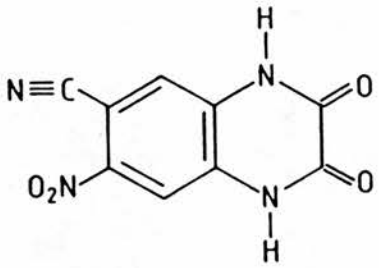
Kynurenate



7-chloro-kynurenate



HA966



CNQX

FIGURE 7 : Chemical structures of compounds with actions at the NMDA-associated glycine site

Abbreviations: HA-966, 3-amino-1-hydroxypyrrolidin-2-one; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione

at this glycine site is L-689,560 which has been used successfully to characterise the glycine site in both rat and human tissue (Grimwood *et al*, 1991a; Gulati *et al*, 1991). The tritiated radioligand shows a similar pharmacological profile to [^{3}H]glycine (Grimwood *et al*, 1991b).

NMDA receptors expressed in *Xenopus* oocytes from rat brain mRNA have revealed an absolute requirement of glycine for activation (Kleckner and Dingledine, 1988). Glycine has also been proposed as the modulating substance responsible for altering the relative affinities and proportions of agonist and antagonist binding at the neurotransmitter recognition site (Monaghan *et al*, 1988; Monaghan and Cotman, 1989). Glycine is thought to promote an agonist preferring conformational state of the receptor but the agonist/antagonist specific distribution of binding sites is still maintained even in the presence of glycine or HA-966 (Monaghan and Cotman, 1989). It is now suggested that there are two anatomically distinct binding sites which display differing states depending on glycine interactions.

It is very difficult to estimate the glycine level in vivo especially in electrophysiological experiments. Ascher and Johnson (1989) have proposed two theories concerning the effect of glycine in vivo. Firstly, the glycine site may be permanently saturated due to high endogenous levels of glycine. Endogenous modulation would therefore only be due to other compounds active at the site (e.g. serine). The second theory is that levels of glycine lower than required for saturation are present normally and that fluctuations in the extracellular concentration can modify synaptic transmission. It is thought that the first theory is most viable since glycine levels in cerebrospinal fluid (CSF) indicate concentrations above 1μM (Skilling *et al*, 1988). Further supportive evidence comes from the fact that whole cell recordings from mammalian brain slices are not modulated by glycine whereas NMDA responses in torn-off patches are modulated by glycine (Llano *et al*, 1988). This would tend to disagree with Monaghan *et al* (1988) who suggest regional variations in glycine concentrations, as seen by the modulation of [^{3}H]L-glutamate binding. The theory of

Monaghan *et al* (1988) is supported by Thomson *et al* (1989) in electrophysiological studies. However, this effect may be selective for different CNS regions (Thomson, 1989).

The NMDA glycine site may prove to be important therapeutically as a control site for decreasing or increasing NMDA receptor activity and glycine antagonists provide a novel angle for modulating NMDA responses.

The Zinc site

The presence of zinc has been demonstrated autoradiographically using $^{65}Zn^{2+}$ in synaptic boutons in the mossy fibre of the CA3 region of the rat hippocampus (Crawford and Conner, 1972). K^+-evoked release of Zn^+ in rat hippocampus CA3 cells has shown that enough zinc is released to have an effect at the NMDA receptor *in vivo* (Aniksztejn *et al*, 1987). Like Mg^{2+}, Zn^{2+} has been shown to antagonise the effects of NMDA by decreasing the channel open time and the channel conductance in cultured cortical and hippocampal neurones (Westbrook and Mayer, 1987; Peters *et al*, 1987). This effect does not show voltage dependence suggesting that Mg^{2+} and Zn^{2+} are acting at two distinct sites within the NMDA receptor complex. Zn^{2+} also inhibits [^{3}H]-TCP binding in a non-competitive manner (Reynolds and Miller, 1988a,b) and decreases NMDA-induced toxicity in cultured neurones (Peters *et al*, 1987). These studies have led to the conclusion that zinc is not interacting with any of the identified sites on the NMDA receptor complex, and that it is acting at a site outwith the channel (Kushner *et al*, 1988).

The tricyclic antidepressant (TCA) class of drugs has been demonstrated to have an effect similar to Zn^{2+} on NMDA responses (Reynolds and Miller, 1988a,d). They demonstrated that the TCA's and derivatives inhibited [^{3}H]dizocilpine binding in a non-competitive manner. They do not compete with glycine or NMDA thereby ruling out these domains as sites of action. Reynolds and Miller (1988d) have concluded that the TCA's may cause their actions at the NMDA receptor via the same site as Zn^{2+}.

The physiological relevance of this Zn^{2+}/TCA site is still unknown but it is clear that Zn^{2+} and the TCA's decrease NMDA induced neurotoxicity in cultured cells (Leander *et al*, 1989) a role that may be of use therapeutically.

The Polyamine site

The polyamines spermine and spermidine potentiate [^{3}H]dizocilpine and [^{3}H]TCP binding to rat synaptosomal membranes. Spermidine increased the affinity of the binding of [^{3}H]dizocilpine from 45nM to 15nM. In the presence of L-glutamate and glycine, spermidine further increased the affinity of [^{3}H]dizocilpine binding from 45nM to 2.2nM and 8.4nM respectively (Ransom and Stec, 1988; Williams *et al*, 1990). Polyamines were found to enhance [^{3}H]CPP binding at high concentrations (Carter *et al*, 1990). Further studies also revealed that polyamines enhanced binding of [^{3}H]glycine resulting in the K_d value being altered from 189nM to 76nM (Ransom and Deschenes, 1990). As yet the functional significance of these polyamine effects is unclear but they are thought to act at a novel site in the NMDA receptor complex. In addition arcaine, a competitive antagonist of the polyamine site has been identified (Reynolds and Miller, 1990).

Since the polyamines are concentrated intracellularly, it seems most likely that the binding site is located on the intracellular surface of the NMDA receptor protein (Fig.4). Gangliosides have also been proposed as having an effect at an intracellular site coupled to the NMDA receptor (Lodge *et al*, 1989). The ganglioside GT1b (trisilalosylgangliotetraglycosylceramide) has been reported to decrease L-glutamate induced cell death in neuronal cultures. A decrease in $^{45}Ca^{2+}$ uptake suggests that GT1b may be having an antagonistic effect at the NMDA receptor (Manev *et al*, 1989). This effect may be due to a novel intracellular interaction with the NMDA receptor since classical Ca^{2+} channel blockers are ineffective as are the NMDA antagonists. The phenolethanolamine ifenprodil was originally described as a cerebral vasodilator although it actually constricts isolated pial vessels (Cook and James, 1981). Ifenprodil and its analogue SL82.0715 have now been described as novel antagonists at the

NMDA receptor. A variety of techniques have been employed to demonstrate this, both *in vivo* and *in vitro* (Carter *et al*, 1988; Gotti *et al*, 1988). Both compounds displace [^{3}H]CPP from its binding site in rat brain membranes with IC_{50} values of 0.1 and 0.3μM for ifenprodil and SL82.0715 respectively. Slightly higher concentrations also partially displace [^{3}H]TCP binding and antagonise the stimulatory effect of L-glutamate on such binding (Carter *et al*, 1989). These effects are all due to non-competitive antagonism. Both drugs also decrease the volume of damage seen in rat and cat brain after a focal ischaemic insult (Carter *et al*, 1988). This is an effect very similar to that seen with TCP. Available evidence suggests therefore that ifenprodil and SL82.0715 are pure-competitive antagonists acting solely at the polyamine site, although their actions are modulated by the polyamines. Ifenprodil may prove useful in the characterisation of the mechanism of action of the polyamines (Williams *et al*, 1991).

1.5 EAA RECEPTORS IN THE DEVELOPING CNS

CNS Development

As an animal develops postnatally its nervous system must change and adapt in order to maintain efficient function. The development of the mammalian CNS is an ordered process and can be divided into four well defined phases (Kellaway, 1989).

1. Functional neurones are formed by differentiation from primitive neuroblasts.
2. Cellular contacts are formed when the differentiated neuronal elements move from the site of genesis to appropriate loci.
3. Anatomic circuitry appropriate to an adult CNS is formed.
4. Functional transmission points within these circuits are formed.

CNS development is species dependent. For instance at birth the rat CNS is a very immature structure while the human CNS is quite well formed. Whatever its state at birth, the mammalian CNS continues to develop through these ordered phases until a fully functional brain emerges. Neurones may gain or lose connections or alternatively the structure and function of synapses may alter during maturation. The adult brain

itself is not a finished product of this processing since it itself is in a continual state of cytoarchitectural flux (Purves and Hadley, 1985; Purves, 1986). Plasticity in adulthood is thought to be essential for processes such as learning and laying down memory (Chang and Greenough, 1984; Lee *et al*, 1980).

The developing brain is very susceptible to influences from both endogenous and exogenous sources, including other neurones, non-neuronal cells and other environmental factors (Ribchester, 1986). The influences may lead to many changes in brain structures such as alteration of neuronal sensitivity or modification of protein synthesis resulting in changes in neuronal structure, circuitry or activity. These influences on the brain will generally and ultimately manifest themselves in the behaviour of the animal. The period of life when animals are most susceptible to such defects coincides with the period of fastest neuronal growth (Cragg, 1975; Hubel and Wiesel, 1970).

Developing neurones require neurotrophic factors to promote their viability. Nerve growth factor (NGF) is necessary for promoting cell survival and for the extension of axons and dendrites. Neurotransmitters released from nerve terminals acting through defined receptors have also been shown to have neurotrophic properties (Cowan *et al*, 1984). Mattson *et al*, (1988) put forward a hypothesis for the involvement of neurotransmitters in neuronal development and degeneration based on findings with the neurotransmitter L-glutamate. Their hypothesis states that:

"Neurodegeneration can result from the overactivity of a brain process (L-glutamate transmission) which at lower levels of activation is involved in normal developmental and functional events."

Apart from L-glutamate other central neurotransmitters have also been implicated in similar processes namely acetylcholine (ACh), serotonin (5-HT) and dopamine (DA) (see Lipton and Kater, 1989).

Detection of L-glutamate/L-aspartate in the developing brain

The presence of the excitatory amino acids L-glutamate and L-aspartate has been demonstrated in the developing brain. The levels of L-glutamate and L-aspartate in cerebral cortex show differing time course profiles during postnatal development (Erdö and Wolff, 1990b). Whereas L-aspartate is present at very low concentrations at postnatal day 2 (PND2), rising gradually during development and peaking maximally at PND30, L-glutamate at PND2 is already present at 50% of adult concentrations. L-Glutamate levels rise from PND2 peaking maximally at PND16. Both L-aspartate and L-glutamate are present at similar levels in adulthood. The developmental profile of L-aspartate shows a great deal of similarity to the postnatal formation of synaptic junctions as described by Aghajanian and Bloom in 1967. These results may therefore indicate that Erdö and Wolff (1989) were measuring neurotransmitter L-aspartate whereas the high level of L-glutamate detected may incorporate both the neurotransmitter and metabolic pools. However, it should be noted that regional differences in the postnatal development of amino acid content formation of synaptic junctions are likely to exist.

High affinity uptake, as already mentioned in Section 1.2, is a good indication of the presence of neurotransmitters. The presence of high-affinity uptake systems very early in life may act as a controlling mechanism to limit precocious or ectopic development of synapses (Fosse *et al*, 1989). Studies of [^{14}C]glutamine uptake during postnatal development showed regional variations in the developing rat hippocampus (Richter and Wolf, 1990). However, this pattern corresponded well to the uptake profile seen using [^{3}H]L-glutamate (Schmidt and Wolf, 1988). Both showed low levels of uptake in newborn animals with a marked enhancement of uptake in hippocampal neuropil areas during the first postnatal weeks. These developmental profiles for EAA uptake systems correlate well with the development of enzymes involved in glutamatergic neurotransmission such as phosphate activated glutaminase (Wolf *et al*, 1988), L-glutamate dehydrogenase and L-aspartate amino transferase, enzymes responsible for metabolising L-glutamate, (Rothe *et al*, 1983; Schmidt and

Wolf, 1987). The development of high-affinity uptake of [^{3}H]L-aspartate has also been studied in the cat visual system (Fosse *et al*, 1989). Regional variations were seen between the specific areas under study and as in the rat the development of the uptake system closely paralleled postnatal changes in synaptogenesis and axonal/dendritic arborization (Winfield, 1981; Cragg, 1975).

It therefore seems that the postnatal increases in L-aspartate and possibly L-glutamate levels closely parallel the development of associated factors required to class these substances as neurotransmitters (see Orrega, 1979), as well as the cytoarchitectural development of the brain.

Ontogeny of EAA receptors

Ligand binding and autoradiography studies have been directed towards ontogenic measurements of the EAA receptors. Over the last decade a number of ligands have been utilised to measure EAA receptors in various brain regions.

(i) Cerebellum

The first reported studies of [^{3}H]L-glutamate binding during postnatal development used rat cerebellar membranes (de Barry, 1980 and Slevin and Coyle, 1981). Both studies found similar developmental profiles for [^{3}H]L-glutamate binding, with binding peaking at PND20 and concluded that this was due to a change in receptor density rather than a change in affinity over time. These studies presumably measured [^{3}H]L-glutamate binding to many sites and not to specific receptors since the original proposal of three EAA receptor subtypes was only being forwarded at this time (Watkins and Evans, 1981). Slevin and Coyle (1981) measured [^{3}H] KA binding and found a similar developmental profile to [^{3}H]L-glutamate, although KA sites developed at a slower rate. The early appearance of KA sites supported previous observations that iontophoretic application of KA to cerebellar neurones was neurotoxic (Seil *et al*, 1979), although this finding had been proposed as a non-receptor mediated phenomenon.

(ii) Hippocampus

Baudry *et al* (1981), using Na^+ independent [^{3}H]L-glutamate binding, examined the ontogeny of L-glutamate binding sites in hippocampal membranes at various developmental stages. Binding increased gradually from PND4 to adult levels, reaching half maximal binding by PND13. They had already reported that Ca^{2+} could stimulate Na^+ independent [^{3}H]L-glutamate binding to adult hippocampal membranes (Baudry and Lynch, 1979) but could only detect Ca^{2+} stimulation from PND9. Adult levels however were reached by PND16. These changes were all due to alterations in receptor number and not to affinity changes. Interestingly they also found that LTP could only be induced in CA1 cells from PND9 and was not apparent prior to this age. Cl^-/Ca^{2+} dependent [^{3}H]L-glutamate binding sites have since been equated with the L-AP4 receptor (Foster and Fagg, 1984). This may therefore have been measured in this study. These results may also indicate similar underlying mechanisms controlling LTP and Ca^{2+} stimulation.

Ben-Ari and co-workers have performed extensive studies comparing the development of rat and human hippocampal EAA receptors using [^{3}H]KA (Ben-Ari *et al*, 1984; Represa *et al*, 1986) and NMDA-sensitive [^{3}H]L-glutamate binding (Tremblay *et al*, 1988; Represa *et al*, 1989) as markers for KA and NMDA respectively. They have found transient increases in KA sites in the stratum lucidum and the supragranule layer of the *fascia dentata* at birth which fall to adult levels of approximately 73% and 50% respectively in human but not in rat hippocampi. KA binding sites were maximal in man at foetal week 25 (FW25) before falling to adult levels of approximately 60% of this maximum. NMDA sites both in rat and human hippocampi also showed transient increases with maximum binding being detected at PND8 (rat) and FW22 - 23 (human). Adult levels were 30% and 45% of this maximum in human and rat respectively. In all cases changes observed were due to changes in receptor number rather than affinity.

Transient expression of [^{3}H]L-glutamate binding has also been observed in the globus pallidus of developing human brain an area which in the adult has no known glutamatergic innervation (Greenamyre *et al*, 1987; Barks, 1988). This site was insensitive to KA, AP7 and NMDA suggesting the transient expression of AMPA or metabotropic receptors.

The exact physiological relevance of transient expression of EAA receptors is unclear, but due to their involvement in neuronal growth and development, which will be operating at peak levels of activity during very early life, it seems reasonable to propose that EAA receptors are important in developmental plasticity during synaptogenesis.

(iii) Other brain regions

The developing visual cortex is particularly malleable during the early postnatal period. Development is subject to use-dependent modifications. This has been demonstrated in the classic experiments of Hubel and Wiesel (1970). Bode-Greuel and Singer (1989) have measured NMDA receptors in the developing cat visual cortex autoradiographically using AP5 sensitive [^{3}H]L-glutamate binding. Binding increased from birth until PND14-28. Binding remained high throughout the second and third month of life falling to adult levels of approximately 50% maximum binding levels. This is an indication that the NMDA receptor may be involved in synaptic plasticity in the kitten visual cortex. An earlier study measured Na^+ independent [^{3}H]L-glutamate binding in visual structures from rat brain (Schliebs *et al*, 1986). Throughout development the lateral geniculate nucleus (LGN) displayed highest levels of binding. The LGN, visual cortex and superior colliculus had similar development patterns. Initially binding was high, peaking at PND15 then falling to adult levels at PND25. Frontal cortex and retina had a similar profile to the previous mentioned structures but peaked maximally at PND10. This binding cannot be equated to any particular EAA receptor but it does provide an indication of differential development in the visual system perhaps highlighting the importance of these structures in synaptic plasticity.

Erdö and Wolff (1990a) have investigated the postnatal development of distinct EAA receptors in rat visual cortex. [^{3}H]CPP binding was low at birth rising to peak at PND14 - 21 with a slow decline up to the age of one year. In contrast both KA and AMPA (measured with [^{3}H]KA and [^{3}H]AMPA respectively) sites were high at birth reaching a peak at PND6. KA and AMPA binding both reached adult levels of approximately 80% and 50% of maximum binding at PND21-28. [^{3}H]KA binding was higher than [^{3}H]AMPA binding. As in the cerebellum and hippocampus changes were all due to receptor number rather than affinity with the exception of AMPA which showed an alteration in receptor affinity as well as in receptor density throughout development. A similar situation was found in the cerebral cortex of rats for [^{3}H]KA and [^{3}H]AMPA binding this may indicate a role other than in synaptic transmission (Erdö and Wolff, 1989). An early study of Na^+ independent [^{3}H]L-glutamate binding to rat cerebral cortical membranes during development gave a biphasic profile (Sanderson and Murphy, 1982). The first peak was at PND20 - 25 before falling off at around PND30 before the second rise to adult levels. This effect cannot be assigned to any specific receptor so it must be assumed to be a summation of all L-glutamate sites.

Electrophysiological studies

Electrophysiological studies have shown that the immature CNS is at all stages an entirely different brain from that of the adult. Electroencephalogram (EEG) can provide an overview of the differences in electrical activity between the mature and immature brain. Noebels (1989) states that "EEG recordings obtained from the immature human CNS would be considered sufficiently abnormal if derived from the scalp of an adult to indicate imminent demise". The ionic dependence of action potentials also alters postnatally in some cortical neurones, with shifts from Ca^{2+} to Na^+ selective currents with similar changes seen in relation to K^+ channels (Mori-Okamoto *et al*, 1983). The hippocampus is a seizure-prone brain structure. A brief seizure can set in motion a cascade of events including neural growth and formation of new synaptic contacts (Ben-Ari and Represa, 1990). Ben-Ari and co-workers have

studied the electrophysiological properties of immature rat hippocampal CA3 pyramidal cells. Using intracellular recording they have revealed the involvement of NMDA receptors in bursting phenomena.

During PNW1 they consistently detected spontaneous giant depolarizing potentials (GDP) in CA3 cells (Corradetti *et al*, 1988). This spontaneous bursting could be blocked or reduced by AP5 and CPP, thus indicating NMDA receptor involvement. The GDP's disappeared during the second postnatal week. This time scale agrees with that of Tremblay *et al*, (1988) who demonstrated a high level of binding to NMDA receptors during the first postnatal week which was followed by a decrease during the second postnatal week. Further work actually showed the GDP's to be mediated by GABA released from interneurones under NMDA receptor control (Ben-Ari *et al*, 1989). This was demonstrated by the ability of picrotoxin and bicucculine to block the GDP's. The actual mechanism of this phenomena is unknown but may be due to an immature excitatory state of the GABAergic system. This may not mature until the second week of life (Cherubini *et al*, 1991), thus reverting to exerting an inhibitory influence as reflected by the decrease seen in the frequency of GDPs. Gaiarsa *et al* (1990) have shown that modulation of the NMDA response by effects exerted at the glycine modulatory site on these receptors also influences the GDP's. Glycine and D-serine cause an increase in GDP frequency but only until PND10 an effect which is blocked by AP5. Co-application of NMDA and glycine has a stimulatory effect blocked by 7-Clkyn. This effect again falls off during the second week of life. These findings indicate that the glycine site on the NMDA receptor is not only present but is also functional from a very early stage of postnatal development.

Other interesting observations made by the same group are that immature CA3 cells have a different current voltage relationship from adult CA3 neurons (Ben-Ari *et al*, 1988). NMDA stimulation does not invoke a region of negative slope conductance on immature I/V plots. This would seem to suggest that the voltage dependent Mg^{2+} block as seen in adults (Nowak *et al*, 1984; Mayer *et al*, 1984) is less consistently present in immature cells. Following NMDA receptor activation, more Ca^{2+} will

therefore be able to flow through the channel which may be used to promote growth cone development (Kater *et al*, 1988) and increase synaptogenesis. This effect has recently been confirmed in hippocampal CA1 cells by two groups (Bowe and Nadler, 1990; Morrisett *et al*, 1990). Both groups although using different recording techniques, demonstrated that Mg^{2+} was less active in young rats than in adults as an antagonist of NMDA induced depolarisations. Ben-Ari's group has also shown that NMDA receptors are present from PND1 and therefore presumably develop prenatally (King *et al*, 1989). CA3 cells became more sensitive to NMDA as measured by burst firing from PND1 - PND10.

Few electrophysiological experiments involving developmental aspects of EAA receptors have been carried out in other regions of the brain. Hamon and Heinneman (1988) have looked at developmental changes in the hippocampus but in the CA1 subpopulation of cells. They found by measuring $[Ca^{2+}]$ decreases extracellularly that the NMDA receptors in striatum pyramidale are most sensitive to NMDA during the age range PND5 - 9 and that between ages PND12 - 30 receptors in the stratum radiatum were most sensitive indicating that pyramidal cell dendrites became more sensitive to NMDA with increasing age, perhaps by an increase in receptor number or by a maturational aspect of receptor development. LTP in CA1 cells has been shown to be reliably detected from PND8 (Baudry *et al*, 1981) but to be at maximal levels at PND15 (Harris and Teyler, 1984). There may therefore be a correlation between receptor number, receptor sensitivity and induction of LTP in the CA1 region of the hippocampus.

The visual system

The role of EAA receptors in the plasticity of the developing visual system has received much attention in the past few years. Until the second or third month of life (the "critical" period), cells in kitten striatal cortex are responsive to visual stimulation through either eye (Wiesel and Hubel, 1965). During the "critical" period binocular connections are readily modified by visual experience (Hubel and Wiesel, 1970).

NMDA receptors are implicated in synaptic plasticity not only in the hippocampus (Collingridge *et al*, 1983) but also the visual cortex (Artola and Singer, 1987). The role of the NMDA receptor in experience dependent plasticity in the visual cortex has been examined. Kittens were subjected to intracortical infusions of AP5 during the "critical" period. Visual responses in 71% of cortical cells were suppressed compared with 33% in adult cats. It was suggested that the NMDA receptor participates in synaptic responses of visual cortical neurones in kittens but not so readily in cats. Results using kynurenate also suggest the involvement of a non-NMDA receptor component in visual system plasticity (Tsumoto *et al*, 1987). These findings resulted in the hypothesis that "NMDA receptors in the visual cortex of young kittens were more effective than those of adult cats" (Artola and Singer, 1987).

Similar findings were reported in kitten monocular deprivation studies. Activation of the NMDA receptor in the visual cortex is important for synaptic plasticity dependent on visual experience with the study also proposing the important role of Ca^{2+} in plasticity (Kleinschmidt *et al*, 1987). By infusing AP5 into the left visual cortex (the right receiving saline) after monocular deprivation, Gu *et al* (1989) were able to prevent the vision dependent recovery of deprived pathways. These studies highlight the importance of NMDA receptor activation in the developing CNS, in particular, the necessity for NMDA receptor activation in the facilitation of use-dependent modifications of synaptic circuitry.

1.6 SUMMARY OF STUDY OBJECTIVES

The first aim of this study was to establish radioligand binding assays which could be used to examine the neurotransmitter binding site and the associated ion channel of the NMDA receptor in tissue obtained from mature and immature rats. [^{3}H]CPP, [^{3}H]D-AP5 and [^{3}H]dizocilpine were used, all having a high affinity and specificity for their respective binding sites.

The interaction between the glycine site and the neurotransmitter site is still poorly understood. Glycine is thought to regulate agonist and antagonist states of the NMDA receptor (Monaghan *et al*, 1988). The effects of glycine, HA-966 and 7-Clkyn, all compounds previously demonstrated as having activity at the glycine site (Foster and Kemp, 1989; Kemp *et al*, 1988), have been investigated on the binding of [^{3}H]CPP and [^{3}H]D-AP5. The findings are discussed in relation to both known electrophysiological and biochemical effects of the compounds, and are compared with reciprocal binding studies.

The main objective of the study was however to use the two main radioligand binding assays ([^{3}H]CPP and [^{3}H]dizocilpine) to create ontogenic profiles of their respective binding sites, the neurotransmitter site and the ion channel site, on the NMDA receptor complex. The NMDA receptor is clearly important in CNS development, however, no study yet has investigated the postnatal development of sites on the NMDA receptor using radioligand binding to the same membrane preparation with radioligands of high specificity and affinity for individual sites. Modulation of [^{3}H]dizocilpine binding by L-glutamate and glycine throughout postnatal development was also examined. This was to determine not only whether these sites were present and functionally coupled early in life but also to see if they behaved as has been previously demonstrated in the mature brain. These findings along with those of functional studies may help to establish the therapeutic usefulness of NMDA antagonists in pathological conditions which are thought to occur early in life as a consequence of NMDA receptor activation. The findings are compared and related to those of other EAA receptor ontogeny studies both biochemical and electrophysiological, as well as with the known postnatal profile of NMDA-induced excitotoxic damage.

CHAPTER 2

METHODS

2.1 INTRODUCTION

The technique of radioligand binding has been used in the present study to investigate the NMDA receptor. Paton and Rang (1965) pioneered this technique. They investigated the binding of [^{3}H]atropine to membranes prepared from guinea-pig ileum. Although most of the resultant binding was actually to non-muscarinic sites some muscarinic sites were in fact specifically labelled. Radioligand binding very simply involves the incubation of a radiolabelled ligand with an homogenate prepared from the tissue under study. The ligand is usually the putative neurotransmitter under study or an agonist/antagonist derivative of a neurotransmitter. Antagonists are preferable to agonists because they generally have a higher affinity and higher specificity for discrete receptor types (Olverman *et al*, 1988). Ideally the radioligand should be highly selective for the system under investigation, radiolabelled to a high specific activity and have a long half life, hence [^{3}H] and [^{14}C] are ideal radioisotopes to use. Taking these factors into account enables the use of low concentrations of ligand since high affinity sites will be saturated at nanomolar concentrations and only a small fraction of the ligand will be bound (Hrdina, 1986). The availability of biologically active compounds labelled to a high specific activity has since led to important advances, not only in the understanding of drug-receptor/neurotransmitter-receptor interactions at the molecular level and the mechanisms underlying the effects but also in receptor purification, distribution and structure-activity studies. This technique has led to the discovery of high-affinity binding sites in relevant tissues for known psycho-active drugs such as the benzodiazepines, opiates, neuroleptics and the antidepressants.

2.2 ANIMALS USED

Male Cob (Wistar) rats of 10-12 weeks of age were routinely used. In the developmental study male Cob rats of 90 days of age were used as adult comparisons, to tissue obtained from Cob pups of either sex (PND0-PND28). The day of birth was regarded as PND0. Adult and pup brains were treated identically.

2.3 MEMBRANE PREPARATION

Two membrane preparations were used in these studies. The preparation of whole membranes is described in Fig.8 and of synaptosomal membranes in Fig.9.

Whole membranes (adapted from Biziere *et al*, 1980)

Rats were killed by stunning and decapitation, pups by decapitation only. The whole brain was then carefully but rapidly removed and placed in ice-cold 0.9% (w/v) saline solution. The appropriate brain areas were dissected out on an ice-cold block and blotted dry. This tissue was then homogenised in 40 volumes (w/v) ice-cold assay buffer in a teflon-glass homogenizer, 3mm clearance, 12 strokes at 800 rpm. The homogenate was centrifuged at 50,000g for 10 min at 4°C in an ultracentrifuge (Centrikon T-2070). The supernatant was discarded and the resultant pellet was resuspended in 40 volumes ice-cold assay buffer and incubated at 35°C for 30 min followed by centrifugation at 50,000g for 10 min at 4°C. This was followed by a further two washing steps, i.e. resuspension in 40 volumes buffer followed by centrifugation at 50,000g. The final pellets were then either resuspended in an appropriate volume of assay buffer for use as fresh membranes or resuspended in 10 volumes of assay buffer and frozen at -20°C until the day of the assay. On the day of the assay frozen membranes were thawed slowly on the bench at room temperature before increasing the volume from 10 to 40 volumes with assay buffer. The membranes were centrifuged at 50,000g for 10 min at 4°C before the final resuspension into assay buffer. For adult whole membranes the final resuspension resulted in approximately 10mg of original tissue being added to each test incubation tube. Pup membranes were prepared in an identical manner but the final resuspension volume was adjusted to give a suitable protein concentration according to age.

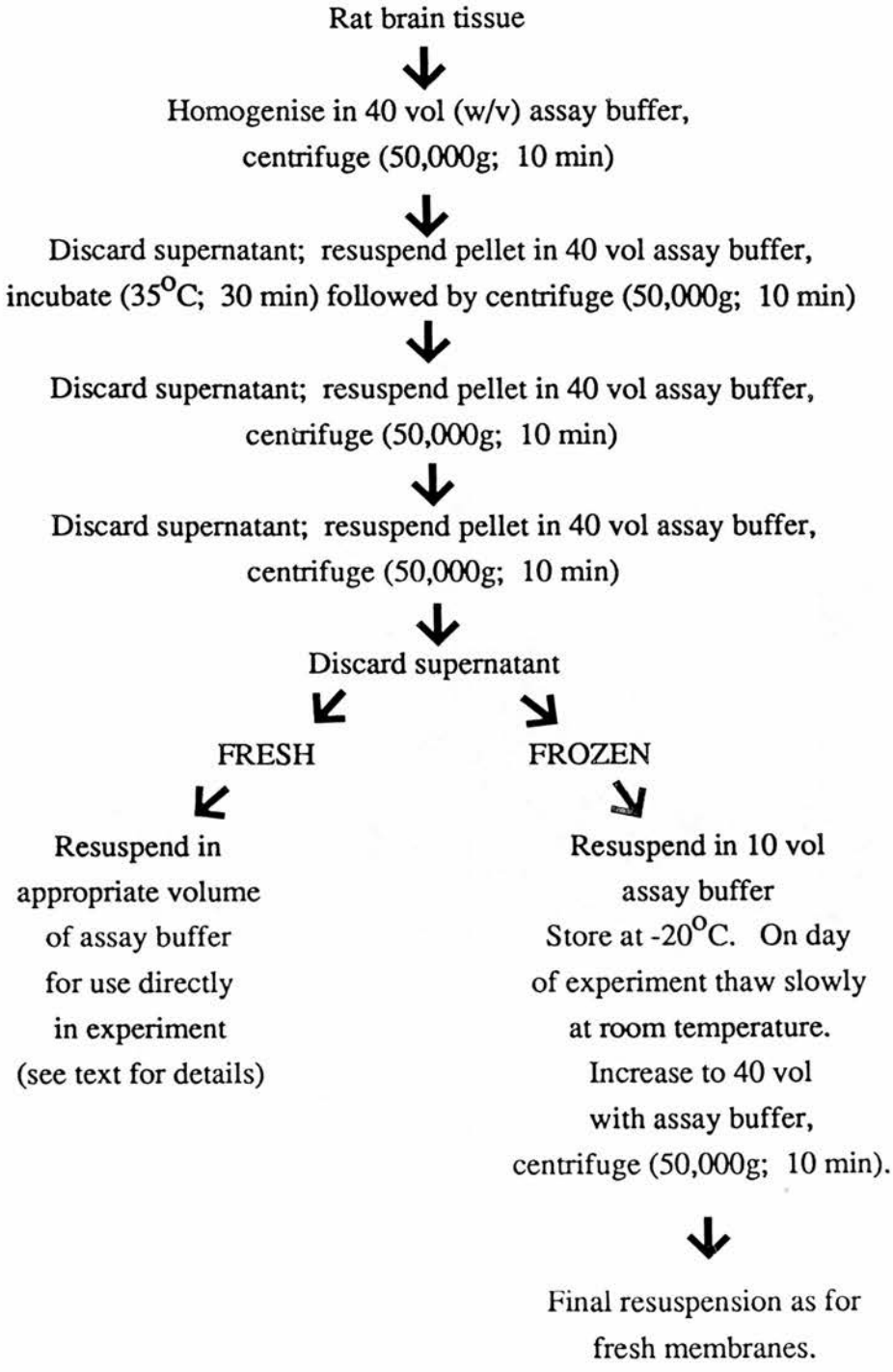


FIGURE 8 : Flow diagram to illustrate the routine preparation of whole membranes from rat tissue

Preparation of membranes subjected to extensive washing included three extra washing steps prior to use either fresh or previously frozen.

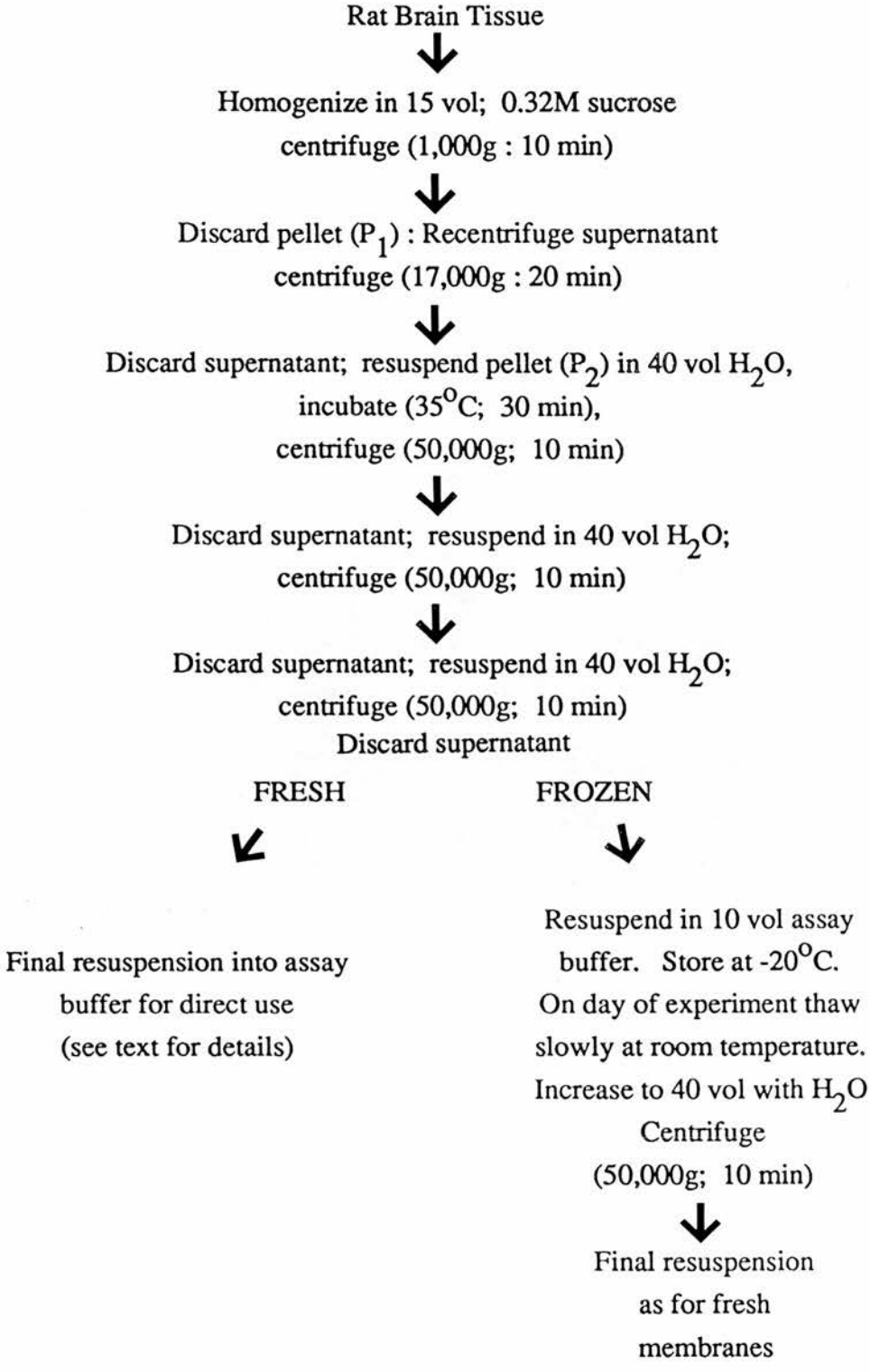


FIGURE 9 : Flow diagram to illustrate the routine preparation of synaptosomal membranes from rat tissue

Preparation of membranes subjected to extensive washing included three extra washing steps prior to use either fresh or frozen.

Synaptosomal membranes (Olverman *et al*, 1984)

Rats were killed by stunning and decapitation, pups by decapitation only. The whole brain was then carefully and rapidly removed and placed in ice-cold 0.9% saline solution. The appropriate brain areas were then dissected out on an ice-cold block and blotted dry. The tissue was homogenised in 15 volumes (w/v) of ice-cold isotonic sucrose (0.32M) using a teflon-glass homogenizer, 3mm clearance; 12 strokes at 800rpm. The resulting homogenate was centrifuged at 1000g for 10 min at 4°C in a bench-top centrifuge (Burkard Koolspin). The supernatant was carefully decanted off and recentrifuged at 17,000g for 20 min at 4°C in the bench-top centrifuge. The P_1 pellet containing blood vessels and myelin was discarded. The resultant pellet (P_2) was then resuspended in 40 volumes of distilled and deionised water and incubated at 35°C for 30 min followed by centrifugation at 50,000g (Centrikon ultracentrifuge) for 10 min at 4°C. The membranes were washed a further twice in distilled and deionised water (40 volumes) and centrifuged (50,000g; 10 min; 4°C). The final resuspension was into assay buffer. In the filtration and centrifugation assay approximately 16mg of original tissue was added to each test incubation tube. Pup membranes were prepared in an identical manner but the final resuspension was adjusted to give a suitable protein concentration according to age.

2.4 RADIOLIGAND BINDING ASSAYS

Centrifugation Assay

Binding assays were routinely performed in 0.7ml polythene micro-centrifuge tubes (Sarstedt). Membranes were added to the tubes containing 50µl aliquots of buffer or drugs under test and allowed to incubate in a water bath at optimum assay temperature for 3 min before addition of a 50µl aliquot of radiolabelled ligand resulting in a final assay volume of 500µl. Following an incubation period during which the binding process reached equilibrium the process was terminated by high-speed centrifugation (15,000g) for 1.5 min in a maxi-fuge (Whyteleaf). The clear supernatant was carefully aspirated off with a fine pasteur pipette and the pellets were washed

rapidly with cold distilled water. The insides of the micro-centrifuge tubes were carefully wiped dry using cotton buds to remove any excess radioactivity without touching the pellet. Each tube containing a pellet was placed in a scintillation vial (Packard). The pellet was then solubilised using concentrated formic acid (100µl). Following the addition of liquid scintillation fluid (3.5ml; Unisolve E, Koch-Light) bound radioactivity was measured in a liquid scintillation analyser (Packard 1900CA).

Filtration assay

Binding assays were routinely performed in 5ml plastic test tubes (Sterilin). Membrane aliquots were added to tubes containing 100µl aliquots of drugs or buffer and allowed to incubate in a water bath at the assay temperature for 3 min before addition of a 100µl aliquot of radiolabelled ligand. The final assay volume was 1000µl. After an incubation period during which the binding process reached equilibrium the process was terminated by rapid filtration over glass fibre filters (Whatman GF/B) using a 24 well cell harvester (Brandel Gaithersburg M.D.). The filters were washed with two 5ml aliquots of buffer. The whole filtration process took 10-15 secs. Filters were then placed in scintillation vials (Packard). Concentrated formic acid (100µl) was added to each filter to solubilise the trapped proteins before addition of liquid scintillation fluid (2ml; Unisolve E, Koch Light). Bound radioactivity was measured in a scintillation analyser (Packard 1900CA).

Routine Binding Assays

Except where indicated binding assays were performed as follows:-

(i) [^{3}H]CPP and [^{3}H]D-AP5

Binding of [^{3}H]CPP and [^{3}H]D-AP5 was routinely carried out using a centrifugation assay. Synaptosomal membranes (0.4ml; 25 vol made up in 50mM Tris-HCl buffer; pH 7.75) were incubated in triplicate at 25^{o}C for 25 min before the

binding reaction was terminated. The volume and concentration of membranes was altered depending on drugs and/or buffer which were to be included in the assay. All further steps are fully described in the section "Centrifugation assay".

(ii) [^{3}H]Dizocilpine

Binding of [^{3}H]dizocilpine was routinely performed using a filtration assay. Whole membranes (0.6ml; 80 vol made up in 5mM Tris-HCl buffer; pH 7.4) or synaptosomal membranes (0.6ml; 37.5 vol made up in 5mM Tris-HCl; pH 7.4) were incubated in duplicate at 25^{o}C for 45 min prior to termination of the binding reaction. As previously the volume and concentration of membranes was altered depending on the amount of drugs, buffer or modulating agents which were to be included in the assay. All further steps are fully described in the section "Filtration Assay".

2.5 PROTEIN ASSAY

Protein concentrations for each membrane preparation used were determined to account for daily variations. The method of Bradford (1976) was used. This assay eliminates the problem of interference by Tris and other reagents encountered in the standard procedure of Lowry *et al* (1951). The reagent used is Coomassie Brilliant Blue G-250 which exists in red and blue forms. Upon binding of the dye to protein the red form is converted to the blue form. This reaction takes place within 2 min and is stable for 60 min. Briefly, samples of membrane homogenate (100µl) were incubated at room temperature for 20 min with protein reagent (5ml), (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid) in disposable cuvettes (BDH). Absorbence was measured at 595nM in a Cecil spectrophotometer. A standard curve was constructed using bovine serum albumin as the standard protein and a new curve was constructed for each assay and each new batch of protein reagent using a multipurpose line-fitting program (Polyfit). Absorbence readings from the membrane preparations were then converted to mg of protein by reference to the standard curve.

2.6 PURIFICATION OF RADIOLIGANDS

Even under recommended storage conditions radioligands are extremely susceptible to decomposition. Therefore regular checks must be made on the state of the radioligand. [^{3}H]D-AP5 has been reported to decompose such that a radioactive impurity, more acidic than D-AP5, interfered with [^{3}H]D-AP5 binding (Olverman *et al*, 1988). The method for purifying and thus separating [^{3}H]D-AP5 from the [^{3}H]impurity is now described, as is a method for purifying [^{3}H]CPP. [^{3}H]Dizocilpine (NEN-DuPont) was bought in quantities such that it lasted for three to six months, after which a new batch was purchased.

[^{3}H]CPP

Unlabelled CPP was synthesised in the laboratory of Dr J.C. Watkins, University of Bristol (Davies *et al*, 1986). The [^{3}H]CPP used in the current study was obtained by custom tritiation of an unsaturated precursor of CPP (Amersham International, Bucks., UK). This was stored in distilled and deionised water under liquid nitrogen (-180^{o}C) pending purification on Dowex AG-1 acetate ion-exchange resin. The resin was purchased in the chloride form and was prepared for use in the acetate form. Fresh resin (5ml) contained within a glass column was prepared by washing with 2M sodium acetate (105ml) until no chloride was detected. The resin was then eluted with distilled and deionised water (35ml) before NaOH (M, 0.5ml) was added to the resin. The column was further eluted with distilled and deionised water (45ml) until a neutral pH was reached. [^{3}H]CPP (2.5μCi in 2.5ml distilled and deionised water) was added dropwise to the resin followed by distilled and deionised water (2.5ml). The resin was further eluted with distilled and deionised water (35ml) and fractions (5ml) were collected. This was to eliminate any [^{3}H]H_2O from the resin. Subsamples were taken for scintillation counting for detection of radioactivity (Packard Tricard 1900CA). The resin was next eluted with 0.1M acetic acid (50ml) to collect [^{3}H]CPP. Fractions (2.5ml) were collected. Subsamples were diluted in distilled and deionised water and taken for scintillation counting. This resulted in one peak of radioactivity being eluted

(Fig.10A). This radioactivity accounted for 76.6% of that added to the column. The fractions corresponding to [^{3}H]CPP were combined and freeze-dried. The residues were resuspended in distilled and deionised water to give a final stock concentration of [^{3}H]CPP. Finally the resin was eluted with acetic acid (40ml) and fractions (5ml) collected. This process should strip the resin of anything remaining on it. Subsamples were taken for scintillation counting.

100μCi of the purified [^{3}H]CPP was added to a fresh Dowex AG-1 acetate resin column. The above process was repeated to confirm that it was authentic [^{3}H]CPP. Again one peak of radioactivity was eluted, corresponding to that in Fig.10A, with 0.1M acetic acid (Fig.10B). This radioactivity accounted for 98% of that added to the column. Aliquots of [^{3}H]CPP (100-200μl) were stored under liquid nitrogen where the ligand remained stable for at least three months. For each binding experiment a fresh aliquot was removed from storage and diluted with assay buffer to the required concentration (usually 100μM) and stored on ice prior to addition to the membrane suspensions.

[^{3}H]D-AP5

Unlabelled D-AP5 was synthesised in the laboratory of Dr J.C. Watkins, University of Bristol (Davies and Watkins, 1982). The [^{3}H]D-AP5 used in the present study was obtained by custom tritiation of an unsaturated precursor of D-AP5 (Amersham International, Bucks, UK). This was stored at a stock concentration of 10mCi/ml in distilled and deionised water under liquid nitrogen (-180^{o}C) pending purification on Dowex AG 50W x 8 ion-exchange resin. The method of Olverman *et al* (1988) was used. Briefly, fresh resin (5ml) contained within a glass column was prepared by washing with NaOH (N, 50ml) followed by distilled and deionised water until a neutral pH was reached. HCl (N, 50ml) followed by distilled and deionised water were then washed through until pH 5 was reached. [^{3}H]D-AP5 (3mCi in 0.5ml distilled and deionised water) was added to the column and the resin eluted with distilled and deionised water (80ml). Fractions (1ml) were collected and subsamples

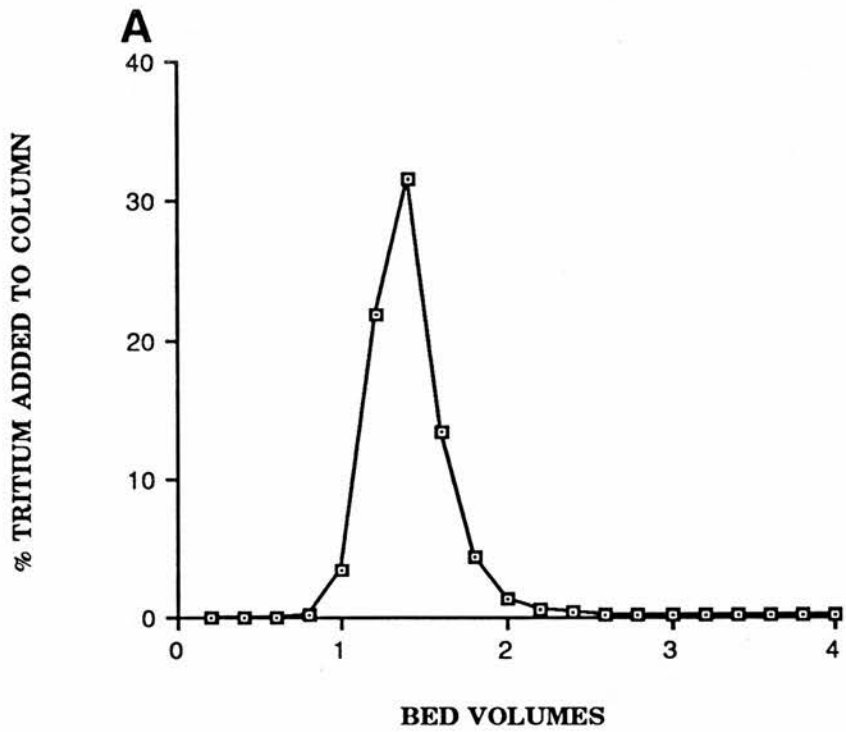
FIGURE 10: Ion exchange purification of [^{3}H]CPP

[^{3}H]CPP was added to a column of Dowex AG-1 ion exchange resin (5ml; 1 bed volume). The resin was prepared for use by elution with 2M sodium acetate(25 bed volumes), distilled and deionised water (7 bed volumes), M NaOH (0.5ml) and finally distilled and deionised water(9 bed volumes). After addition of the unpurified sample of [^{3}H]CPP to the resin it was further eluted with distilled and deionised water (7 bed volumes), 0.1M acetic acid (10 bed volumes) and M acetic acid(8 bed volumes). Fractions (1ml) were collected and the radioactivity in each determined and expressed as a percentage of radioactivity added to the column.

(A). 2.5 mCi of a sample of unpurified [^{3}H]CPP resulted in radioactivity being eluted in one peak by 0.1M acetic acid.

(B). One hundred microcuries of [^{3}H]CPP collected from the peak in (A) above was retested by addition to fresh column of resin. Only one peak was obtained corresponding to that seen in (A) above with 0.1M acetic acid.

In (A) the fractions corresponding to CPP when combined accounted for 76.6% of radioactivity added. In (B) 98% of the radioactivity added was accounted for by CPP.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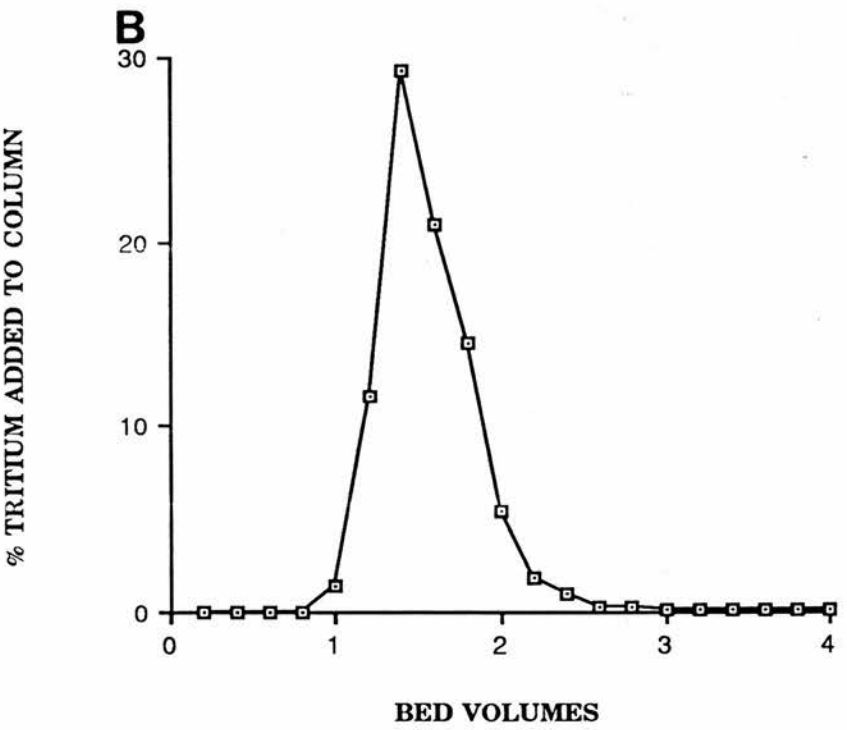
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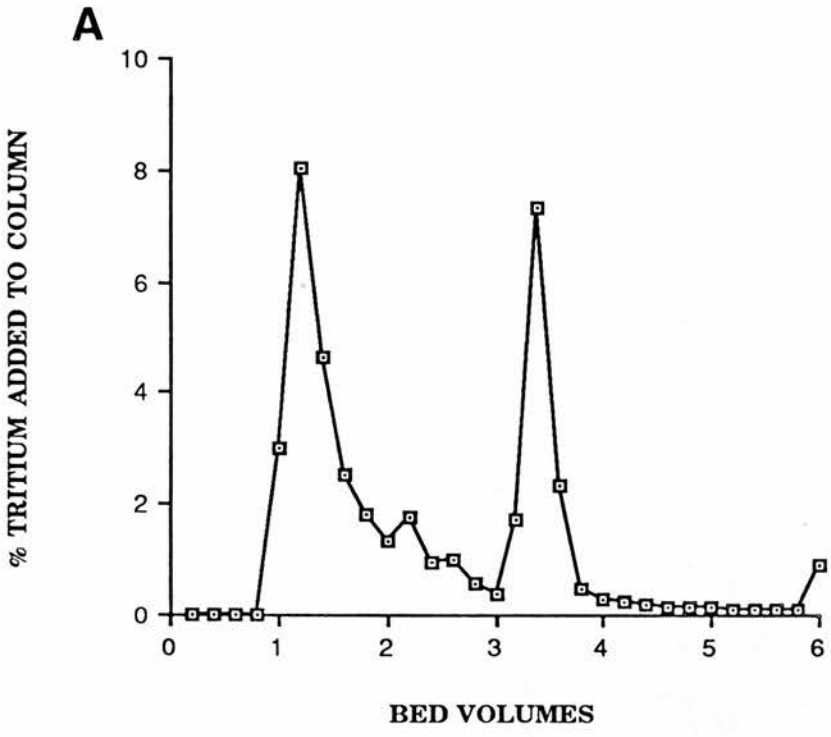
FIGURE 11: Ion exchange purification of [^{3}H]D-AP5

[^{3}H]D-AP5 was added to a column of Dowex AG 50W x8 resin (5ml; 1 bed volume). The resin was eluted with distilled and deionised water (16 bed volumes). Fractions (1ml) were collected and the radioactivity in each determined and expressed as a percentage of radioactivity added to the column.

(A). Three millicuries of a sample of unpurified [^{3}H]D-AP5 resulted in radioactivity being eluted in two peaks with distilled and deionised water.

(B). One hundred microcuries of [^{3}H]D-AP5 collected from the second peak in (A) above was retested by addition to a fresh column of resin. Only one peak was eluted with distilled and deionised water, corresponding to the second peak in (A). The second peak in (A) corresponds to [^{3}H]D-AP5 (Olverman et al 1988) and accounts for 12.8% of radioactivity added to the column. 95% of radioactivity added to the second column (B) was accounted for by D-AP5.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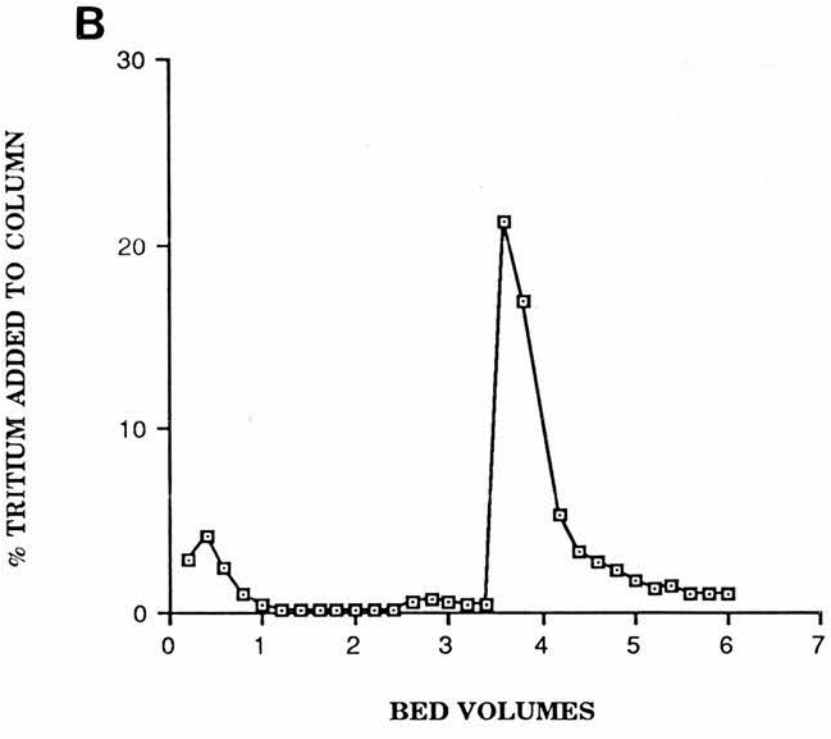
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(1-2µl) were taken for scintillation counting (Packard Tricarb 1900CA). This resulted in two peaks of radioactivity being eluted (Fig.11A). The second peak corresponded to [^{3}HD-AP5 (Olverman *et al*, 1988), and accounted for 12.8% of the radioactivity added to the column. Fractions corresponding to [^{3}H]D-AP5 were combined and freeze dried. The residues were resuspended in distilled and deionised water to give a final concentration of 20µM [^{3}H]D-AP5.

100µCi of the purified [^{3}H]D-AP5 was added to a fresh AG 50W x 8 resin column, and was subjected to the steps described above to confirm that it was authentic [^{3}H]D-AP5. This resulted in one peak of radioactivity being eluted with distilled and deionised water (Fig.11B). This peak corresponded to the second peak in Fig.11A and accounted for 95% of the radioactivity added to the column. Aliquots of [^{3}H]D-AP5 (100-200µl) were stored under liquid nitrogen where the ligand remained stable for at least three months. For each binding experiment a fresh aliquot was removed from liquid nitrogen storage and diluted with assay buffer to the required concentration (usually 200nM) and stored on ice prior to addition to the membrane suspensions.

2.7 DATA ANALYSIS

Disintegration experiments have shown that 1µCi of radioactivity is equivalent to 2.22 x 10^6dpm's. Knowing both this and the specific activity of the radioligand under study (in Ci/mmol), it is simple to transform specific binding (expressed in dpm's) into an amount of ligand specifically bound (moles). This value can be expressed as the number of moles per weight of tissue or protein to take into account variations in membrane preparations between experiments.

Kinetic parameters of K_d (receptor affinity) and B_{max} (number of receptors present) were calculated. A computer curve fitting programme was used to fit raw experimental data. ("Polyfit", an IBM multipurpose programme written by Dr. R.B. Barlow; see Bowmer, 1992). This allowed consistent handling of complex binding data. This program allows the fitting of values of Y by the least squares method to

values of X. The logistic expression below (Equation 1) was used where: Y = response, X = ligand concentration, M = maximum effect and, IC_{50} is the concentration of inhibitor producing half maximum effect and P defines the slope.

$$Y = \frac{MX^P}{(X^P + IC_{50}{}^P)} \qquad \text{Equation 1}$$

When X is plotted logarithmically the curve is S-shaped, with a slope determined by P. It can be used therefore to assess whether results obey the Law of Mass Action. If they do P should be equal to 1. In inhibition studies P is negative with the curve being "reverse S-shaped" relating the amount of ligand bound to varying concentrations of competing unlabelled ligand. P is therefore equivalent to n_H, the Hill Co-efficient, where n is the slope of the line at the midpoint (Barlow and Blake, 1989). The Hill Co-efficient is an indication of the existence of co-operativity. A Hill number of close to unity indicates either a lack of co-operativity or binding site heterogeneity.

The dissociation constant K_i was calculated graphically in some instances using the findings of Cheng and Prussoff (1973; Equation 2) where [L] = ligand concentration, IC_{50} is the concentration of inhibitor producing half maximal effect and K_d in the dissociation constant.

$$Ki = \frac{IC_{50}}{1 + [L]/K_d} \qquad \text{Equation 2}$$

When assay conditions are such that $[L]/Kd << 1$ then $Ki \sim IC_{50}$. Ligand-receptor interactions are saturable therefore a finite number of receptors must exist in a given tissue. The term B_{max} is used to define the maximum number of receptor sites present in a tissue. Therefore if specific binding follows the Law of Mass Action the relationship between the amount of ligand bound (SB) and ligand concentration [L] is as shown in Equation 3.

$$SB = \frac{Bmax\,[L]}{[L] + Kd} \qquad \text{Equation 3}$$

A plot of SB/[L] against SB gives a straight line from which both B_{max} and K_d can be calculated (Scatchard Plot), but this method was not routinely used in the present study.

Statistical Analysis

Mean values and standard error of mean (s.e.m.) were calculated where appropriate. Developmental data were subjected to one-way or two-way analysis of variance as appropriate (Monk and Hall, 1980). Analysis of variance (anova) allows the reliability of a difference between means to be assessed in the light of the overall variability of the data. An F ratio was computed from mean squares (C.L.R. Anova; Clear Lake Research Incorporated, 1985; 1986). A low F ratio generally signifies a lack of significance between means. A larger F ratio makes it more likely that a significant effect exists. One-way anova (between subject) can be successfully applied to the data presented in the present study where the groups are of unequal numbers. Differences between ages were investigated using this method. Two-way analysis of variance was used to look for differences between conditions and age. This also indicates whether an interaction is occurring. This refers to the way in which the effects of one independent variable are influenced by the other independent variable.

Since the F ratio gives no indication as to where the difference lies, an a posteriori comparison must be made (i.e. post hoc test). In these studies a t-test was used to locate significant ($p < 0.05$) differences. All possible pairwise comparisons between means were made. Analysis of variance results are expressed as follows: F (degrees of freedom) = x; $p < 0.05$. Student's t-test was applied where appropriate to other data (Statworks^{TM}). Lines of best fit were fitted to data using Polyfit.

Developmental Study

Adult (PND90) comparisons were routinely included in each experiment using immature tissue (PND0-PND28). The reason being to control for the assay itself. If for any reason binding to the PND90 tissue was not as expected the whole experiment was discarded.

Specific binding, K_d, B_{max} and EC_{50} values were calculated, where appropriate, for adult and immature binding and subjected to analysis of variance (as described above) to look for differences between ages. Since data were grouped in age intervals (see Section 3.5.1) any slight variation in receptor affinity or EC_{50} may be undetected using analysis of variance. Therefore two alternative methods were used to investigate the variation in K_d and EC_{50} values. The first simply involved plotting all data points against individual ages and fitting a straight line to them using the method of least squares. The second method involved calculating the ratio of specific binding (SB)/B_{max} and then plotting this against respective age. The ratio should be an indication of K_d since from Equation 4, [L] is fixed, therefore SB/B_{max} must approximate K_d. A straight line was again fitted to these data as above. If the slope of these lines was close to zero it was assumed that no changes were occurring in EC_{50} or K_d postnatally. However, lines with a sharp gradient may be an indication of postnatal alteration. Plots of SB/B_{max} were constructed for binding expressed both per mg protein and per mg tissue, since they were identical, only the former plots shall be mentioned.

2.8 MATERIALS

[^{3}H]MK801 (specific activity 24.8Ci/mmol) was purchased from New England Nuclear (NEN-DuPont). CPP and D-AP5 were synthesised in the laboratory of Dr J.C. Watkins (Department of Pharmacology, University of Bristol). The ligands were prepared by custom tritiation (Amersham International, Bucks) to specific activities of 20Ci/mmole and 40Ci/mmole for [^{3}H]CPP and [^{3}H]D-AP5 respectively. HA966,

AP7 and 7-chlorokynurenate were also synthesised in the laboratory of Dr Watkins. (+)Dizocilpine (MK(+)), (-)dizocilpine (MK(-)) and (±)dizocilpine (MK(±)) were obtained from Fujisawa Pharmaceutical Company (Japan).

All other reagents and compounds used were purchased from routine laboratory suppliers and were of the purest grade available.

CHAPTER 3

RESULTS

3.1 INTRODUCTION

The first objective of this study was to establish binding assays for [^{3}H]CPP and [^{3}H]dizocilpine to the same membrane preparation which could be used to generate reliable information about their respective binding sites on the NMDA receptor complex. The aims were (a) to use the [^{3}H]CPP assay to investigate interactions between the neurotransmitter binding site and the NMDA associated glycine site and (b) to use both radioligand binding assays to study the ontogeny of the NMDA receptor complex in the rat CNS between birth (PND0) and adulthood (PND90).

NMDA receptors are present in highest densities in the hippocampus and the cerebral cortex (Wong *et al* 1986; Monaghan *et al* 1985; Olverman *et al* 1988). Therefore to maximise binding under conditions when it may be very low, such as in neonatal tissue, membrane preparations were prepared from pooled hippocampal and cerebral cortical tissue. Two membrane preparations were investigated; (i) a whole membrane preparation, and (ii) a crude synaptosomal preparation, as described in Section 2.3.

3.2 CHARACTERISATION OF [^{3}H]CPP BINDING

3.2.1 Timecourse of [^{3}H]CPP binding to synaptosomal membranes

Using the standard centrifugation assay (Section 2.4) [^{3}H]CPP (15nM) bound to freshly prepared synaptosomal membranes from rat cerebral cortex. Binding to freshly prepared whole membranes was not successful, resulting in low specific binding making accurate measurements difficult. For this reason synaptosomal membranes were used in all further experiments concerned with characterising [^{3}H]CPP binding. At 25^{o}C [^{3}H]CPP (15nM) binding was rapid, reaching equilibrium within 5 minutes (Fig.12). The non-specific component of the binding measured with L-glutamate (1mM) was essentially instantaneous varying very little during the incubation. Specific binding at equilibrium was around 180fmol/mg protein, accounting for approximately 40% of total binding. Binding was reversible since addition of L-glutamate (1mM) after 15 min incubation displaced all bound ligand rapidly.

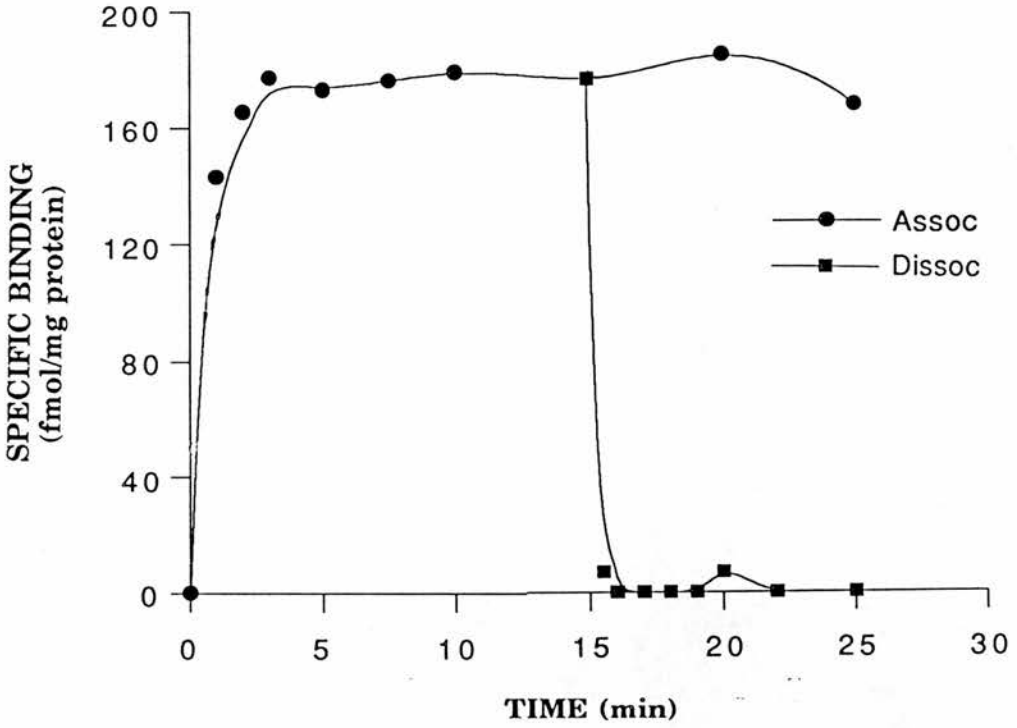


FIGURE 12: Timecourse of [^{3}H]CPP binding

Synaptosomal membranes were incubated with [^{3}H]CPP (15nM) at 25° at pH7.5 using 50mM Tris-HCl for various times(circles; Assoc) in the absence or presence of L-glutamate(1mM) to measure total and non-specific binding respectively. In replicate control samples L-glutamate (1mM) was added after 15min incubation (squares; Dissoc). Specific binding was determined by subtracting non-specific binding from total binding. Specific binding accounted for 40% of total binding. Each point is the mean of triplicate determinations.

3.2.2 The effect of varying experimental conditions on the binding of [^{3}H]CPP

The proportion of total [^{3}H]CPP binding to rat synaptosomal membranes accounted for by specific binding (~ 40%) is relatively low compared with say [^{3}H]dizocilpine binding (see Section 3.3.1). It was therefore important to try and optimise experimental conditions to increase specific binding.

Effect of Ca^{2+}

Inclusion of Ca^{2+} in the assay buffer may result in reduced non-specific binding (Olverman *et al*, 1988). Inclusion of a physiological concentration of Ca^{2+} (2.5mM) in the standard assay buffer (50mM Tris-HCl, pH 7.5) in the form of $CaCl_2$ resulted in specific binding accounting for 31% of total binding. This was no better than in the absence of Ca^{2+} (40%) and was therefore not included in further assays.

Effect of Tris-acetate buffer

50mM Tris-acetate buffer (pH 7.4), was compared with 50mM Tris-HCl buffer (pH 7.5). No differences were seen between the proportion of specific binding under each condition. This lack of effect was further confirmed by calculation of K_d and B_{max} values. K_d values of 0.79μM and 0.63μM were seen with Tris-acetate and Tris-HCl with corresponding B_{max} values of 3.5 and 3.3 pmol/mg protein.

Effect of extra washing of membrane preparations

Extra washes of membrane preparations should lead to a lowering of endogenous neurotransmitters. This should result in improved binding. However longer preparation time may be deleterious to the membranes. Extra washing (x 2 extra washes) of this membrane preparation had no beneficial effects on [^{3}H]CPP binding compared with standard membrane preparation (Fig.9).

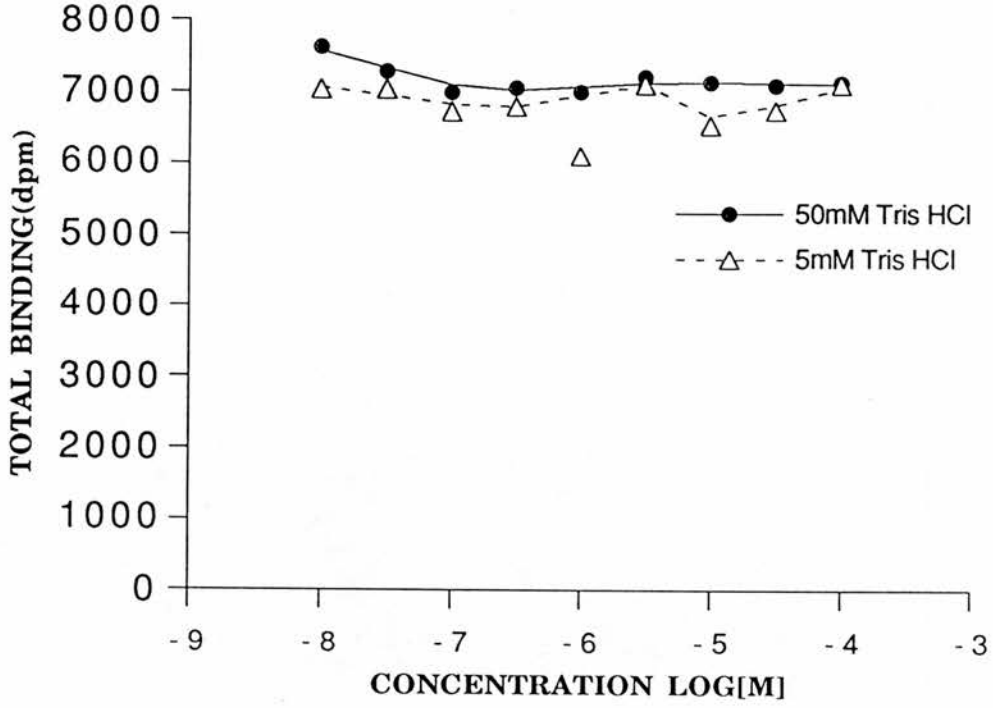


FIGURE 13: Inhibition of [^{3}H]CPP binding using filtration to terminate the assay
Synaptosomal membranes were prepared from pooled cortical and hippocampal tissue and incubated with [^{3}H]CPP (10nM) at 25°C for 25 min using 5mM(circles) or 50mM Tris HCl(triangles), (pH 7.5). Binding was inhibited with increasing concentrations of CPP (0.01-100μM). Non-specific binding was measured with L-glutamate (1mM). The assay was terminated using filtration as described in Section 2.4. Each point represents the mean of duplicate determinations. Non-specific binding using 5mM or 50mM buffer accounted for 95.5% and 91.3% of total binding respectively.

TABLE 3 THE EFFECT OF pH ON THE BINDING OF [^{3}H]CPP

Synaptosomal membranes were incubated at 25°C for 25 min with [^{3}H]CPP (10nM) using 50mM Tris HCl of increasing pH (6.5 - 8.5). Non-specific binding was measured with L-glutamate (1mM). Specific binding was calculated by subtracting non-specific binding from total binding.

pH	Specific Binding (fmol/mg protein)
6.50	14.4 ± 1.4
7.00	37.9 ± 5.7
7.25	50.6 ± 9.8
7.50	54.9 ± 10.9
7.75	82.7 ± 8.6
8.00	45.5 ± 11.1
8.25	34.5 ± 6.1
8.50	18.7 ± 4.5

n = 5 or 6

Values represent mean ± s.e.m.

levels of binding were seen at pH 7.75 at 25°C, with lower binding seen towards the extremes of the pH range. K_d and B_{max} values were calculated for [^{3}H]CPP binding at pH 7.75.

3.3 CHARACTERIZATION OF [^{3}H]DIZOCILPINE BINDING

3.3.1 Timecourse of [^{3}H]dizocilpine binding to synaptosomal membranes

The binding of [^{3}H]dizocilpine (1nM) to synaptosomal membranes prepared from pooled rat cerebral cortices and hippocampi in the presence of L-glutamate and glycine (both 10μM) reached equilibrium by 45min of incubation at 25°C. This level of binding remained stable until at least 60 min of incubation (Fig.14). Non-specific binding was defined using unlabelled dizocilpine (30μM). Although the use of the same compound in unlabelled form is not ideal, no other compound is currently commercially available which shows the same degree of specificity and affinity for the [^{3}H]dizocilpine binding site. At equilibrium, unlike [^{3}H]CPP, specific binding accounted for approximately 92% of total binding.

3.3.2 Determination of K_d, B_{max} and n_H values

K_d and B_{max} values were to be routinely estimated from concentration dependent inhibition curves by fitting data to Equation 1 (Section 2.7). However to confirm that this method was reliable these parameters were also initially calculated using Scatchard and Hill plots (Fig.15). The specific binding in the presence of increasing concentrations of [^{3}H]dizocilpine (0.5 - 10nM) was measured as described in Section 3.4.1. These data were transformed into Scatchard (Fig.15A) and Hill (FIg.15B) plots for the estimation of B_{max}, K_d and n_H values. The K_d calculated from Fig.15A is 4.6nM with a corresponding B_{max} of 1.83pmol/mg protein. The K_d value calculated from Fig.15B is 5.01nM, which correlates well with the K_d value derived from Fig.15A. The n_H value of 1.009 indicates a single population of binding sites. These values compare well with those calculated by fitting binding data obtained by inhibiting [^{3}H]dizocilpine binding with increasing concentrations of unlabelled

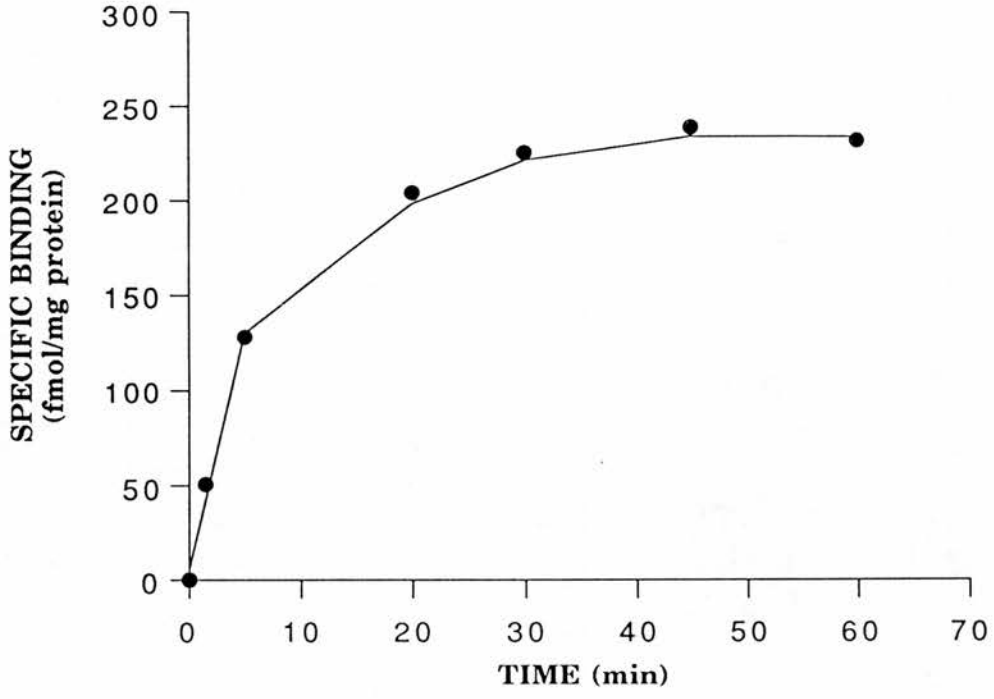


FIGURE 14: Timecourse of [^{3}H]dizocilpine binding

Synaptosomal membranes were prepared from pooled cortical and hippocampal tissue. They were incubated at 25°C with [^{3}H]dizocilpine (1nM) using 5mM Tris-HCl(pH7.4) in the presence or absence of dizocilpine (30μM) for various times to measure non-specific and total binding respectively. Binding was measured in the presence of L-glutamate and glycine (both 10μM). Specific binding was calculated by subtracting non-specific binding from total binding. Each point is the mean of duplicate determinations.

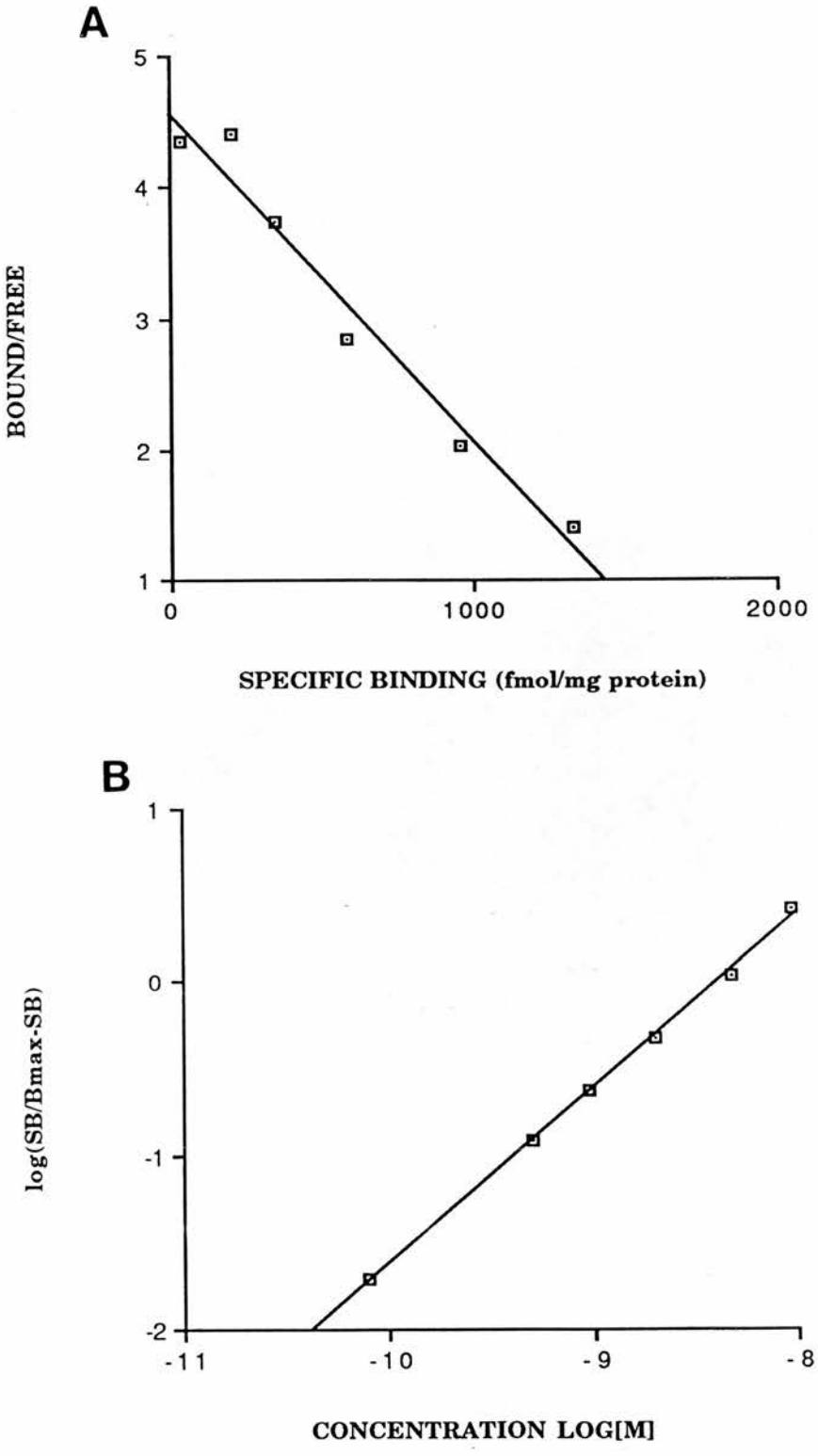


FIGURE 15: Scatchard(A) plot and Hill(B) plot of [^{3}H]dizocilpine binding
Synaptosomal membranes prepared as described in Fig.13 were incubated with increasing concentrations of [^{3}H]dizocilpine (0.5-10nM) for 45min in the absence or presence of dizocilpine (30μM) to measure total and non-specific binding. Binding was measured in the presence of L-glutamate and glycine (both 10μM). Specific binding was calculated and transformed into a Scatchard plot(A), from which a K_d of 4.6nM and a Bmax of 1.83pmol/mg protein were estimated for [^{3}H]dizocilpine binding. Data were further transformed into a Hill plot (B) from which a Hill co-efficient (n_H) of 1.009 was estimated along with an IC_{50} of 5.01nM.
Abbreviation: SB, specific binding

TABLE 4 BINDING CHARACTERISTICS OF [^{3}H]DIZOCILPINE TO TWO MEMBRANE PREPARATIONS

Membranes, normal (3 x washes) or washed (6 x washes), were incubated with [^{3}H]dizocilpine (1nM) for 45 min at 25°C. Binding was measured using either 5mM or 50mM Tris-HCl (pH 7.4). Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated. Replicate samples were incubated with increasing concentrations of dizocilpine (0.01-100nM) or L-glutamate (0.01 - 100μM). Data were fitted to the logistic equation $Y = MX^P/(X^P + IC_{50})$, K_d, B_{max} and EC_{50} values were calculated. Maximal binding was measured in the presence of 10μM L-glutamate. Specific binding at this concentration was expressed as a percentage of control binding.

MEMBRANE PREPARATION	SPECIFIC BINDING (fmol/mg protein)	K_d (nM)	B_{max} (pmol/mg protein)	EC_{50} FOR L-GLUTAMATE (μM)	MAXIMAL BINDING (% control)
5mM Buffer					
Synaptosomal					
Normal	52.34	8.4	0.49	0.29	434
Washed	13.95	77	1.09	0.38	1202
Whole					
Normal	73.46	4.2	0.38	0.35	202
Washed	52.22	69	3.66	0.35	454
50mM Buffer					
Synaptosomal					
Normal	31.40	NM	NM	0.26	286
Whole					
Normal	33.67	NM	NM	0.16	149

NM : not measured

dizocilpine, to the equation $Y = MX^p /(X^p + IC_{50}{}^p)$ (Equation 1). Under identical experimental conditions a K_d of 6.8 ± 1.0nM (n = 13) was calculated with a corresponding B_{max} of 1.7 ± 0.3pmol/mg protein (n = 13) with n_H being 1.1 ± 0.09 (n = 13). This inhibition method was used in all further experiments.

3.3.3 Effects of buffer concentration

Binding of [^{3}H]dizocilpine (1nM) to whole and synaptosomal membrane preparations subjected to normal washing (3 x washes) was investigated in the presence of 5mM and 50mM Tris-HCl (pH 7.4). The results are from a representative experiment whereby a single whole and a single synaptosomal membrane preparation were halved during preparation such that one half was prepared with 5mM buffer and the other half was prepared with 50mM buffer. Binding was inhibited using increasing concentrations of dizocilpine (0.01-100nM), and this data was used to calculate K_d and B_{max} values. L-Glutamate (0.01-100μM) was used to modulate binding and EC_{50} values were calculated for this effect (Table 4).

Control binding

In the presence of 50mM buffer it proved difficult to demonstrate reliable inhibition of [^{3}H]dizocilpine binding. K_d and B_{max} values could not be measured. However, in the presence of 5mM buffer K_d and B_{max} values were calculated for each membrane preparation (Table 4). The K_d value for binding to whole membranes was lower than that for synaptosomal membranes. B_{max} values indicated a higher density of binding sites in synaptosomal membranes than in whole membranes.

L-Glutamate modulation

L-Glutamate modulation of [^{3}H]dizocilpine binding was apparent regardless of the buffer concentration. A concentration dependent increase in specific binding was observed, and maximum binding was measured in the presence of 10μM L-glutamate under both conditions. The ability of L-glutamate to cause this enhancement of

binding as measured by the EC_{50} values, was similar in the presence of 5 or 50mM buffer to both membrane preparations. The percentage increase in specific binding, in the presence of 10μM L-glutamate compared to control binding was however greater in the presence of 5mM buffer and was greatest for synaptosomal membranes(Table 4).

3.3.4 The effects of extensive membrane washing

Extensive washing of a membrane preparation may prove advantageous since a greater proportion of endogenous modulators should be removed. This is important for [^{3}H]dizocilpine binding since it will allow measurements of exogenous modulation to be made. However the extended preparation time may ultimately be harmful to the membrane preparation and may lead to the removal of important cellular elements. The effects of extensive washing (6 x washes) were investigated on the binding of [^{3}H]dizocilpine (Washed membranes; Table 4). Since binding in the presence of 50mM Tris-HCl was unreliable all further assays were carried out in the presence of 5mM Tris-HCl. As in section 3.3.3 results are from the single representative study whereby one whole and one synaptosomal membrane preparation were prepared, with one half subjected to normal washing (3 x; normal membranes, as already described above), and the other half subjected to extensive washing (6 x; washed membranes). K_d, B_{max} and EC_{50} values were calculated as described in Section 3.4.3 and are displayed in Table 4 (washed membranes).

Control binding

Extensive washing decreased the amount of specific binding detected for each membrane preparation compared to normal membranes. The largest decrease in binding was seen for synaptosomal membranes (75%). This effect is further reflected in the calculated K_d values. The K_d values for [^{3}H]dizocilpine binding to both whole and synaptosomal membranes increase with extensive washing indicating that the affinity of [^{3}H]dizocilpine for its binding site has decreased. B_{max} values increase for both membrane types after extensive washing compared to normal conditions.

L-Glutamate modulation

L-Glutamate increased [^{3}H]dizocilpine binding dose-dependently to both membrane preparations with normal and extensive washing. EC_{50} values however did not alter with extensive washing for either membrane preparation, washed or normal. Maximum levels of binding were again seen in the presence of 10μM L-glutamate for each membrane preparation. The extent of the L-glutamate modulation was greater for synaptosomal membranes, both with normal and extensive washing. Greatest modulation was measured in extensively washed membranes.

3.3.5 Effects of freezing and thawing membrane preparations

Freezing membrane preparations prior to use can be advantageous since they can be prepared in advance and stored in usable aliquots. This would be extremely useful for neonatal tissue which was to become available at short notice. In addition freezing and thawing can be beneficial to membrane preparations since this process can allow the disruption of vesicles containing endogenous neurotransmitters and neuromodulators. As described above (Section 3.3.4) one whole and one synaptosomal membrane preparation was prepared. Half was frozen for at least 24 hours prior to use, the other half was used fresh. Binding, examined under control conditions and in the presence of L-glutamate and glycine (both 10μM), was inhibited with increasing concentrations of dizocilpine (0.01-100nM). In addition, binding was positively modulated with increasing concentrations of L-glutamate (0.01 - 100μM) or glycine (0.01-100μM). K_d, B_{max} and EC_{50} values were calculated.

Control binding

Specific binding of [^{3}H]dizocilpine to both membrane preparations which had been previously frozen was reduced compared to fresh membranes (Table 5). Under control conditions K_d and B_{max} values did not vary greatly between fresh and frozen membranes (Table 6). Concentration dependent inhibition of [^{3}H]dizocilpine binding was measured with dizocilpine in both fresh and previously frozen membranes (Fig.16).

TABLE 5 THE EFFECTS OF FREEZE/THAWING ON [^{3}H]DIZOCILPINE BINDING

Membranes, fresh or previously frozen, were incubated with [^{3}H]dizocilpine (1nM) for 45 min at 25°C. Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated. Replicate samples were incubated in the presence of L-glutamate and glycine (both 10μM). Modulated binding was expressed as a percentage of that measured under control conditions.

MEMBRANE TYPE	SPECIFIC BINDING (fmol/mg protein)		MAXIMAL BINDING (% control)
	control	+ L-glu & gly	
FRESH			
whole	51.90	90.91	175
Synaptosomal	45.36	149.38	329
FROZEN			
whole	39.35	191.79	487
Synaptosomal	29.41	431.37	1466

L-glu: L-glutamate (10μM)

gly: glycine (10μM)

TABLE 6 BINDING CHARACTERISTICS OF [^{3}H]DIZOCILPINE TO FRESH AND FROZEN MEMBRANE PREPARATIONS

[^{3}H]Dizocilpine binding was carried out as detailed in Table 5 but in the presence of increasing concentrations of dizocilpine (0.01 - 100nM) in the absence or presence of L-glutamate and glycine (both 10μM). Replicate samples were incubated in the presence of increasing concentrations of L-glutamate or glycine (0.01 - 100μM). Data were fitted to the logistic equation $Y = MX^p/(X^p + IC_{50})$, K_d, B_{max} and EC_{50} values were calculated.

MEMBRANE TYPE	Control		+L-glu & gly		L-glu	gly
	K_d (nM)	B_{max} (pmol/mg protein)	K_d (nM)	B_{max} (pmol/mg protein)	EC_{50} (μM)	EC_{50} (μM)
FRESH						
Whole	8.32	0.48	6.50	0.68	0.10	0.10
Synaptosomal	26.80	1.24	5.21	0.93	0.18	0.11
FROZEN						
Whole	6.21	0.28	2.92	0.75	0.13	0.16
Synaptosomal	39.80	1.2	2.95	1.70	0.28	0.19

L-glu; L-glutamate (10μM)
gly; glycine (10μM)

Modulation of binding

L-Glutamate and glycine each increased the binding of [^{3}H]dizocilpine to whole and synaptosomal membranes, fresh and previously frozen, in a concentration dependent manner with maximal binding being seen at 10μM glycine and at 10μM L-glutamate (Table 5; Fig.16 and 17). L-glutamate enhanced binding to both membrane preparations, fresh and frozen, to a greater extent than glycine (Fig.17). The increase in binding seen with L-glutamate alone was comparable to the increase in binding seen with both L-glutamate and glycine (Table 5; Fig.17).

The EC_{50} values for L-glutamate and for glycine modulation of [^{3}H]dizocilpine binding did not vary greatly between fresh and previously frozen, whole and synaptosomal membranes.

Likewise B_{max} values obtained from previously frozen membranes did not greatly differ from those calculated for fresh membranes in the absence or presence of L-glutamate and glycine. K_d values were reduced in both fresh and frozen membranes in the presence of L-glutamate and glycine (Fig.16; Table 6).

3.3.6 Inhibition of [^{3}H]dizocilpine binding to the two membrane preparations chosen to be used in the present study

The inhibition of [^{3}H]dizocilpine (1nM) binding to fresh synaptosomal membranes and previously frozen whole membranes was investigated in the absence and presence of L-glutamate (10μM). [^{3}H]Dizocilpine ((+)dizocilpine) binding was inhibited with the(+) isomer(MK(+)) the (-)isomer(MK(-)) and compared with the racemic(±) mixture (MK(±)) of dizocilpine. Inhibition curves are displayed in Figs.18 and 19, for whole and synaptosomal membranes respectively, with corresponding K_i values in Table 7. The rank order of potency of these compounds for inhibition of [^{3}H]dizocilpine binding was, MK(+) > MK(±) > MK(-) for synaptosomal membranes and MK(±) > MK(+) > MK(-) for whole membranes. Hill constants are near to unity for each compound in each preparation (Table 7).

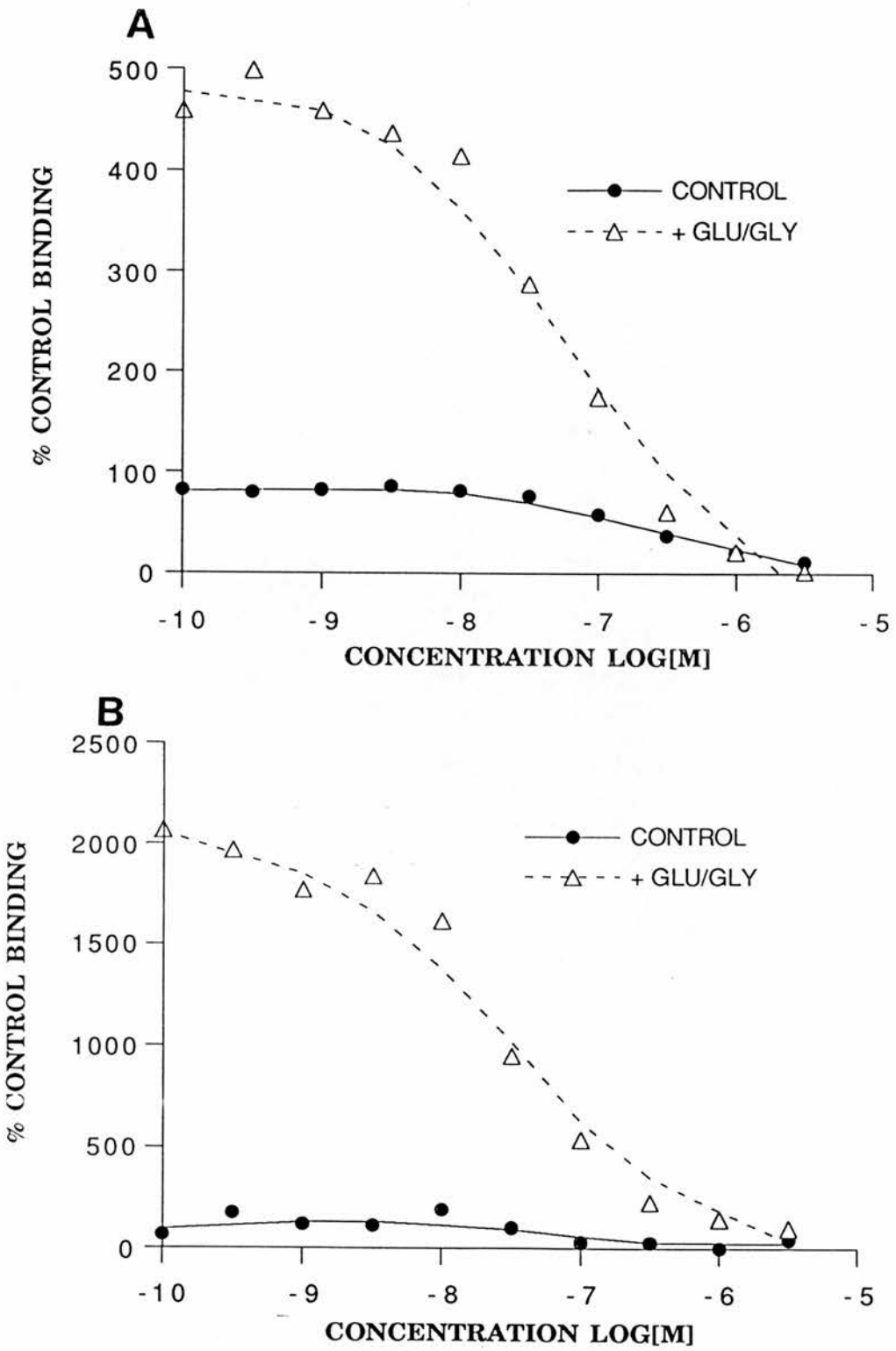


FIGURE 16: Inhibition of [^{3}H]dizocilpine binding to fresh and previously frozen synaptosomal membranes

One membrane preparation was prepared and halved with one half used fresh and the other half used after being frozen for at least 24 hours. Membranes, fresh (A) and previously frozen (B), were incubated with[3H]dizocilpine (1nM) at 25°C for 45min in the presence of increasing concentrations of dizocilpine (0.01-300nM). Binding was measured in the absence(CONTROL) or presence of L-glutamate and glycine(+GLU/GLY;both 10μM). Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated and expressed as a percentage of binding under control conditions. Each point is the mean of duplicate determinations. Abbreviations: GLU, L-glutamate; GLY, glycine.

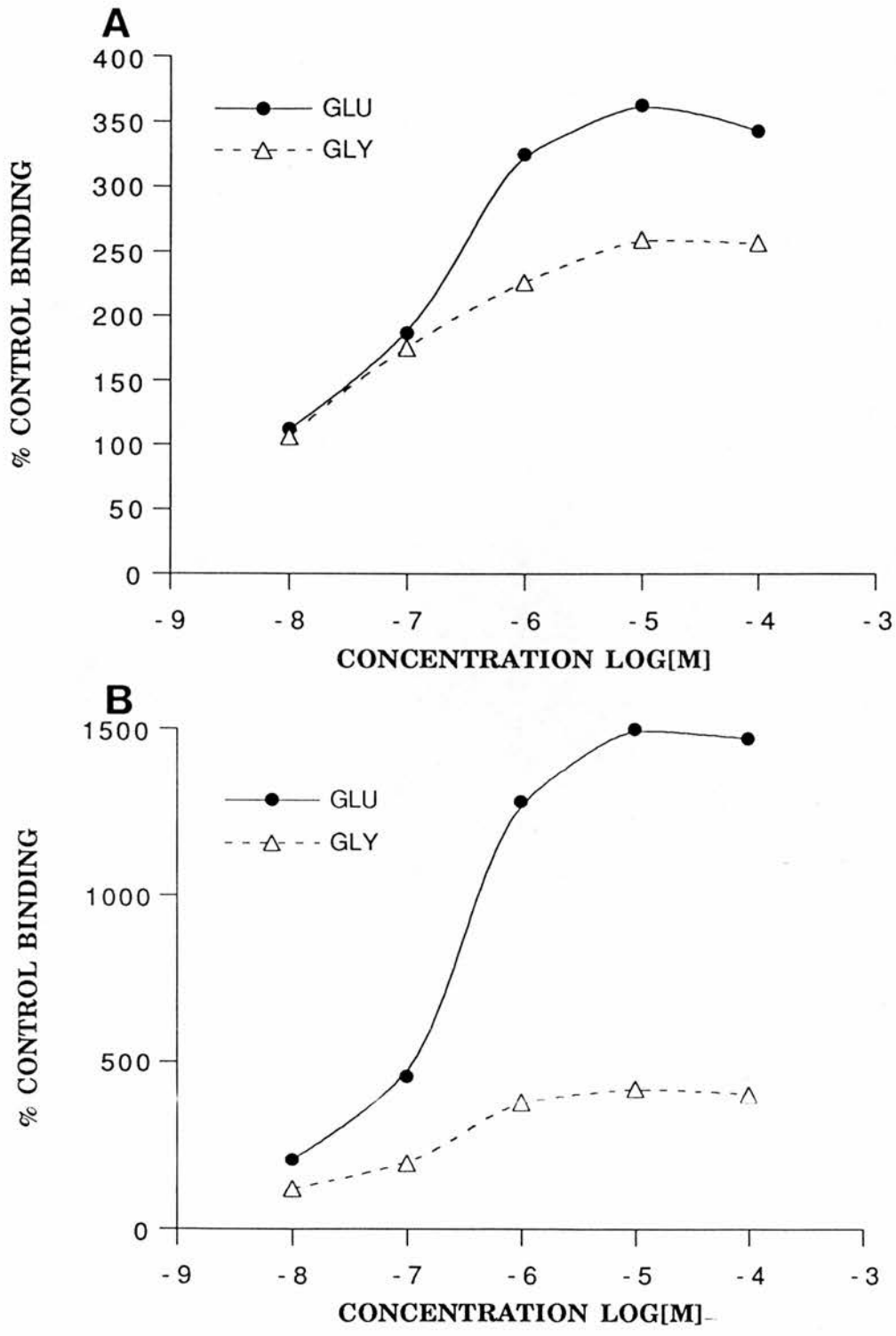


FIGURE 17: Modulation of [^{3}H]dizocilpine binding to synaptosomal membranes
One membrane preparation was used as described in Fig.16. Membranes, fresh (A) and previously frozen (B), were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the presence of increasing concentrations of L-glutamate(GLU) or glycine(GLY);,(0.01 - 100μM). Dizocilpine (30μM) was used to determine non-specific binding. Specific binding under each modulatory condition was calculated and expressed as a percentage of binding under control conditions. Each point is the mean of duplicate determinations.
Abbreviations: GLU, L-glutamate; GLY, glycine.

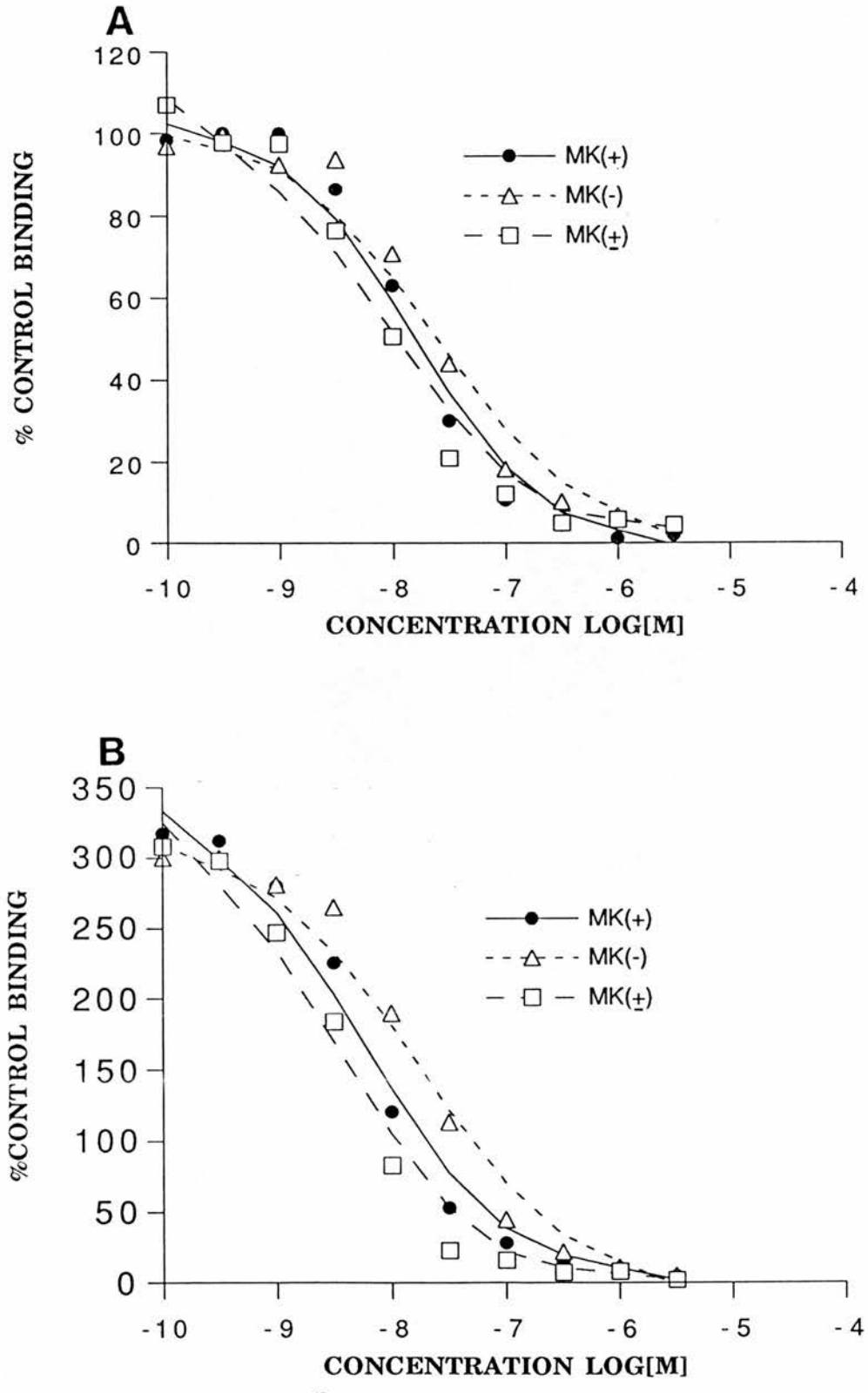


FIGURE 18: Inhibition of [^{3}H]dizocilpine binding to whole membranes
Previously frozen whole membranes prepared from cortical tissue were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min. Binding was inhibited with increasing concentrations of MK(-), MK(+) or MK(±) (see text for explanation of abbreviations), in the absence (A) or presence(B) of L-glutamate (10μM). Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated and expressed as a percentage of binding under control conditions. Data were fitted by least squares to the logistic expression: $Y = MX^p / (X^p + IC_{50})$. Each point represents the mean of two separate observations.

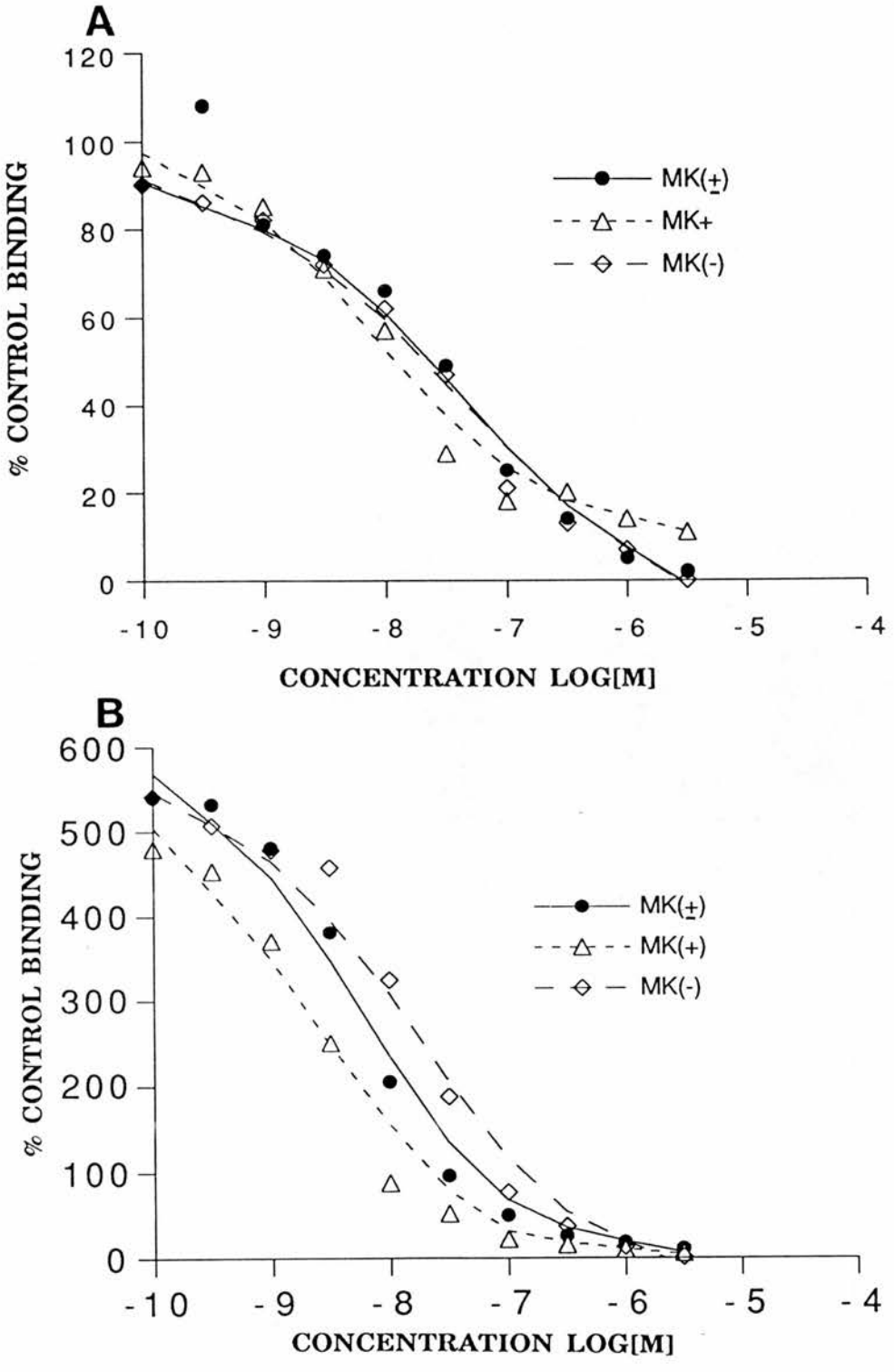


FIGURE 19: Inhibition of [^{3}H]dizocilpine binding to synaptosomal membranes
Fresh synaptosomal membranes prepared from cortical tissue were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min. Binding was inhibited with increasing concentrations of MK(-), MK(+) or MK(±) (see text for explanation of abbreviations), in the absence(A) or presence(B) of L-glutamate (10μM). Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated and expressed as a percentage of binding under control conditions. Data were fitted by least squares to the logistic expression: $Y = MX^P/(X^P + IC_{50})$. Each point represents the mean of two separate observations.

TABLE 7 INHIBITION CONSTANTS FOR THE DISPLACEMENT OF [^{3}H]DIZOCILPINE FROM SYNAPTOSOMAL (A) AND WHOLE (B) MEMBRANES BY STEREOISOMERS OF DIZOCILPINE

[^{3}H]Dizocilpine binding was measured as described in Table 5 to fresh synaptosomal or previously frozen whole membranes, in the presence of increasing concentrations of MK+, MK- or MK± (0.01 - 100nM). Binding was measured in the absence or presence of L-glutamate (10μM). Data were fitted to the logistic equation $Y = MX^p/(X^p + IC_{50})$, K_i values were calculated.

	Control		+L-glutamate (10μM)	
	K_i (nM)	n_H	K_i (nM)	n_H
A: Synaptosomal Membranes				
MK+	9.4	0.96	3.7	0.93
MK-	85.1	0.82	17.9	0.98
MK±	25.6	0.73	6.9	0.96
B: Whole Membranes				
MK+	18.7	0.94	11.3	1.14
MK-	24.0	0.92	17.3	1.02
MK±	14.3	0.99	8.2	0.93

Values are the means of two experiments.

3.3.7 Summary of optimal assay conditions used in all further studies

Since an ontogenic study was to be carried out using both [^{3}H]CPP and [^{3}H]dizocilpine it was important to evaluate binding of both ligands to identically prepared membranes to enable direct comparisons of binding parameters. As [^{3}H]CPP bound most reliably to fresh synaptosomal membranes not subjected to extensive washing this preparation was used in all further [^{3}H]CPP binding studies. The calculated K_d was 0.25μM (0.2, 0.3) with a corresponding B_{max} of 2.6pmol/mg protein (2.2, 2.9). All further binding assays were carried out at 25^{o}C in the presence of 50mM Tris-HCl (pH 7.75) using a centrifugation assay to terminate the binding process.

However, a synaptosomal preparation may not be entirely appropriate for an ontogenic binding study due to the low numbers of synaptic junctions in early life (Aghajanian and Bloom, 1967). Therefore the ontogeny of [^{3}H]dizocilpine binding throughout postnatal development was also investigated using a whole membrane preparation. As freezing and thawing did not adversely influence [^{3}H]dizocilpine binding, previously frozen membranes were used. These were prepared without extensive washing which was shown to alter the B_{max}.

3.4 MODULATION OF [^{3}H]CPP AND [^{3}H]D-AP5 BINDING VIA THE NMDA GLYCINE SITE

3.4.1 Introduction

[^{3}H]CPP and [^{3}H]D-AP5 are specific, high affinity ligands both binding to the NMDA receptor neurotransmitter recognition site (Olverman *et al*, 1988; Murphy *et al*, 1987). It has been reported that glycine is an essential requirement for the activation of the NMDA receptor by L-glutamate (Kleckner and Dingledine, 1988). The mechanism responsible for the link between these two sites is still unknown. Monaghan *et al* (1988) however propose two possibilities, multiple NMDA receptor subtypes or

distinct conformational states, which are regulated by glycine. Studies were carried out to investigate the effects of glycine, HA-966 and 7-Clkyn, compounds which all act at the NMDA glycine site, on the binding of [^{3}H]CPP and [^{3}H]D-AP5.

3.4.2 Inhibition of [^{3}H]CPP binding

[^{3}H]CPP (10nM) binding was inhibited by CPP, L-glutamate and D-AP5 in a concentration dependent manner (Fig.20; Table 8). The calculated K_d for [^{3}H]CPP binding was 0.44 ± 0.04μM with a corresponding B_{max} of 2.68 ± 0.45 pmol/mg protein (n = 5; Table 8).

3.4.3 Inhibition of [^{3}H]D-AP5 binding

[^{3}H]D-AP5 binding was inhibited by L-glutamate, D-AP5 and CPP (Fig.21). The K_d for [^{3}H]D-AP5 binding was 0.82 ± 0.19μM with a corresponding B_{max} of 3.2 ± 0.82pmol/mg protein (n = 3). IC_{50} values of 0.1μM and 0.89μM were calculated for CPP and L-glutamate inhibition respectively.

3.4.4 The effect of glycine

[^{3}H]CPP binding

The effect of increasing concentrations of glycine were investigated on the binding of [^{3}H]CPP. A slight potentiation in the amount of specific binding was measured in the presence of 0.01 - 30μM glycine but at higher concentrations (> 30μM) a slight reduction in binding was seen when compared to control binding (Fig.22). Binding was increased to a maximal level of 120.7 ± 5.1% of control binding in the presence of 3μM glycine. At higher concentrations (> 30μM) binding decreased dose-dependently, reaching 83.3 ± 5.9% of control levels in the presence of 1mM glycine. Glycine therefore exhibits both weak stimulatory and weak inhibitory effects on the binding of [^{3}H]CPP.

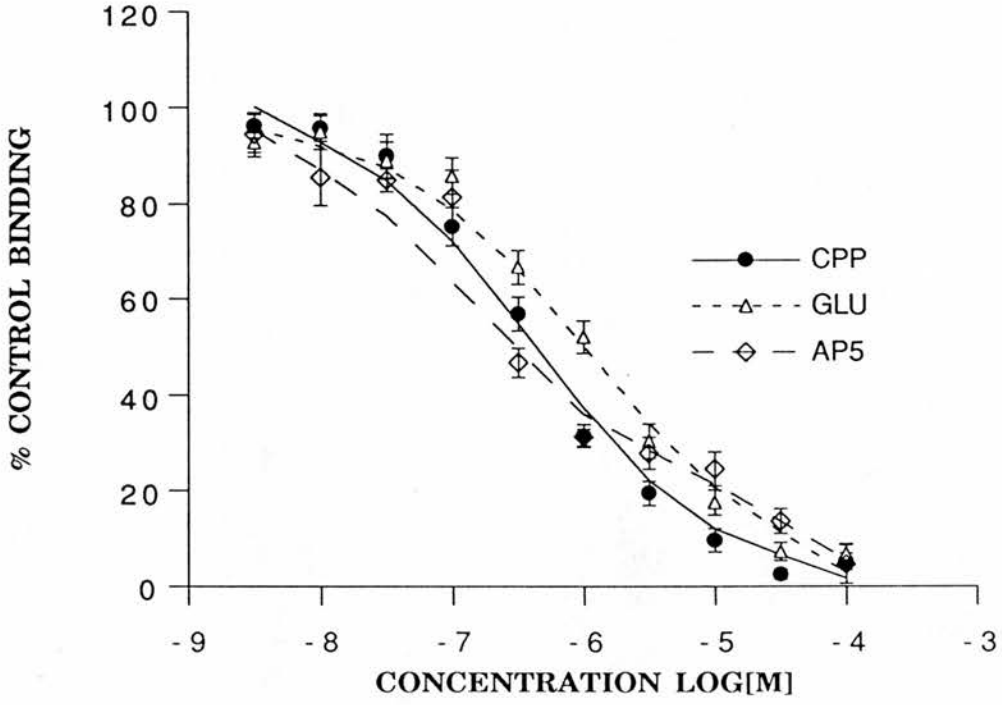


FIGURE 20: Inhibition of [^{3}H]CPP binding

Synaptosomal membranes were incubated with [^{3}H]CPP (10nM) at 25°C for 25 min using 50mM Tris-HCl with increasing concentrations of CPP (n=5), L-glutamate (n = 5; GLU) or D-AP5 (n = 3, AP5), (0.03 - 100μM). Non-specific binding was measured with L-glutamate (1mM). Specific binding in the presence of inhibitor was expressed as a percentage of binding under control conditions. Raw data were fitted by least squares to the logistic expression: $Y = MX^p/(X^p + IC_{50})$, K_d, K_i and Bmax values were estimated.

Each value represents mean ± sem.

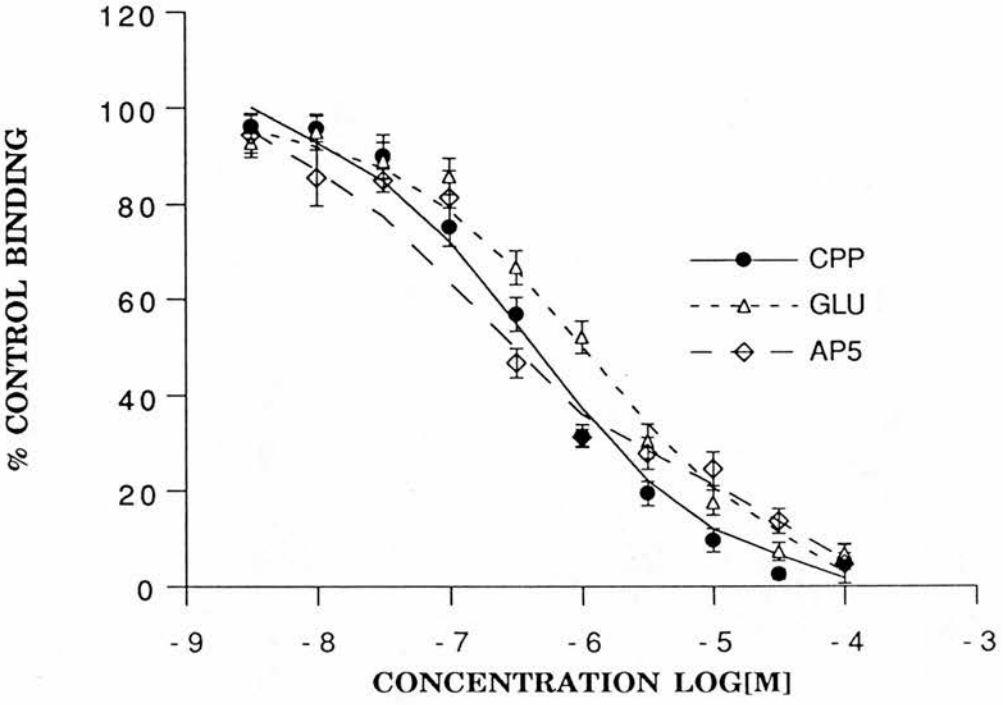


FIGURE 20: Inhibition of [^{3}H]CPP binding

Synaptosomal membranes were incubated with [^{3}H]CPP (10nM) at 25°C for 25 min using 50mM Tris-HCl with increasing concentrations of CPP (n=5), L-glutamate (n = 5; GLU) or D-AP5 (n = 3, AP5), (0.03 - 100μM). Non-specific binding was measured with L-glutamate (1mM). Specific binding in the presence of inhibitor was expressed as a percentage of binding under control conditions. Raw data were fitted by least squares to the logistic expression: $Y = MX^P/(X^P + IC_{50})$, K_d, K_i and Bmax values were estimated.

Each value represents mean ± sem.

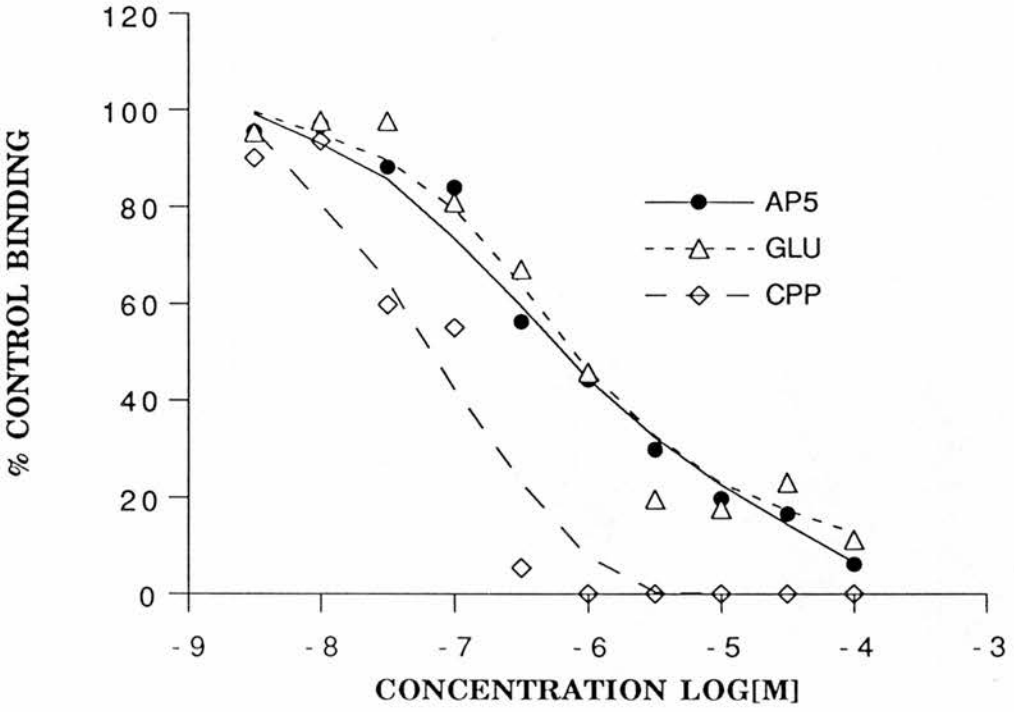


FIGURE 21: Inhibition of [^{3}H]D-AP5 binding to synaptosomal membranes
[^{3}H]D-AP5 binding was measured as described for [^{3}H]CPP binding in Fig.20, in the presence of increasing concentrations of D-AP5(AP5), CPP or L-glutamate (GLU) (0.03-100μM). Non-specific binding was measured with L-glutamate (1mM). Specific binding in the presence of inhibitor was expressed as a percentage of binding under control conditions. Data were fitted by least squares to the logistic expression: $Y = MX^P/(X^P + IC_{50})$, K_d, K_i and Bmax values were estimated. Each value is the mean ± sem of 3 individual experiments for D-AP5 inhibition and 2 experiments for CPP and L-glutamate inhibition.

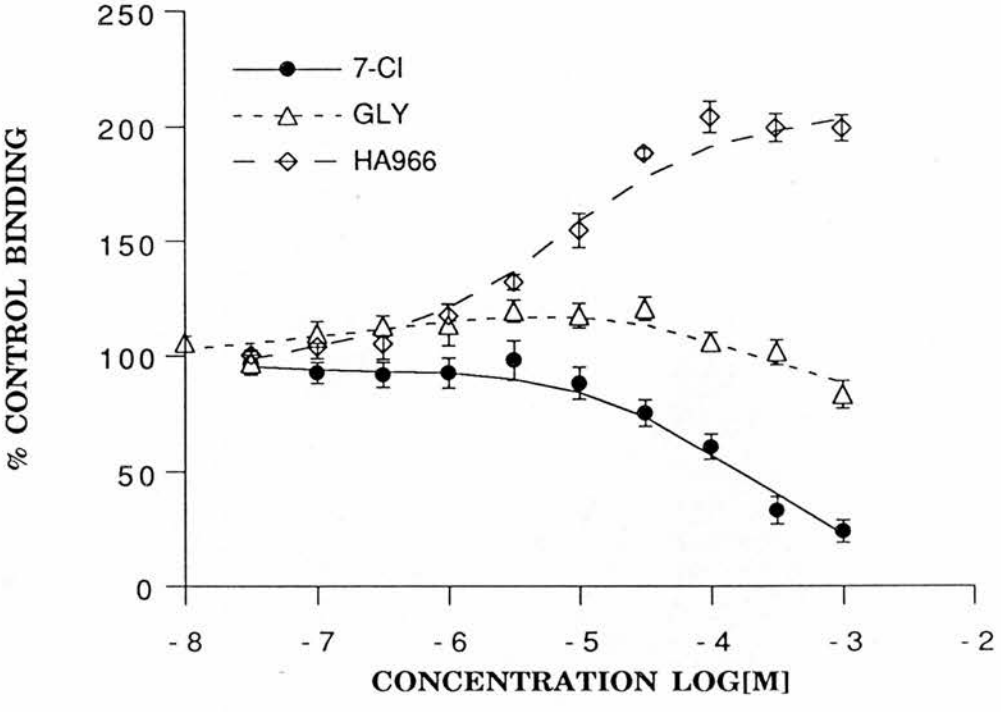


FIGURE 22: The effect of compounds active at the NMDA glycine site on the binding of [^{3}H]CPP

Membranes were incubated with [^{3}H]CPP(10nM) at 25°C for 25 min with increasing concentrations of glycine (n = 5; GLY), HA-966 (n = 5; HA966) and 7-Clkyn (n = 3; 7-Cl), (0.01 - 1000μM). Specific binding in the presence of inhibitor was expressed as a percentage of binding under control conditions. Data were fitted by least squares to the logistic expression: $Y = MX^P/(X^P + IC_{50})$, K_i and EC_{50} values were estimated as appropriate.

Each value represents mean ± sem.

[^{3}H]D-AP5 binding

Increasing concentrations of glycine had a markedly different effect on the binding of [^{3}H]D-AP5 from the effect already described for [^{3}H]CPP binding (Fig.23). Glycine only inhibited [^{3}H]D-AP5 binding. However, glycine could not totally inhibit binding. Maximum inhibition (37.6%) was seen in the presence of 3μM glycine with no further inhibition seen even at higher glycine concentrations. This is a concentration dependent inhibitory action which has an IC_{50} of approximately 0.6μM.

3.4.5 The effect of HA-966

HA-966 has been described as both an antagonist and a partial agonist at the NMDA associated glycine site (see Thomson, 1990).

[^{3}H]CPP binding

HA-966 markedly increased the binding of [^{3}H]CPP in a concentration dependent manner over the concentration range 0.03-1000μM, (Fig.22). In the presence of 100μM HA-966 binding reached a maximum level of 204.5 ± 6.% of control binding. The EC_{50} for this modulating effect was 6.3 ± 1.6μM.

[^{3}H]D-AP5 binding

HA-966, like glycine also had a very different effect on [^{3}H]D-AP5 binding from that seen on [^{3}H]CPP binding. No potentiation of binding was seen. Binding was in fact inhibited (Fig.23). As with glycine binding could not be completely inhibited with around 70% of [3H]D-AP5 binding remaining insensitive to HA-966 inhibition. The IC_{50} for this inhibition is estimated as 5μM.

3.4.6 The effect of 7-chlorokynurenate

7-Clkyn is reported as being either an antagonist or an inverse agonist at the NMDA associated glycine site (Kemp and Foster, 1989).

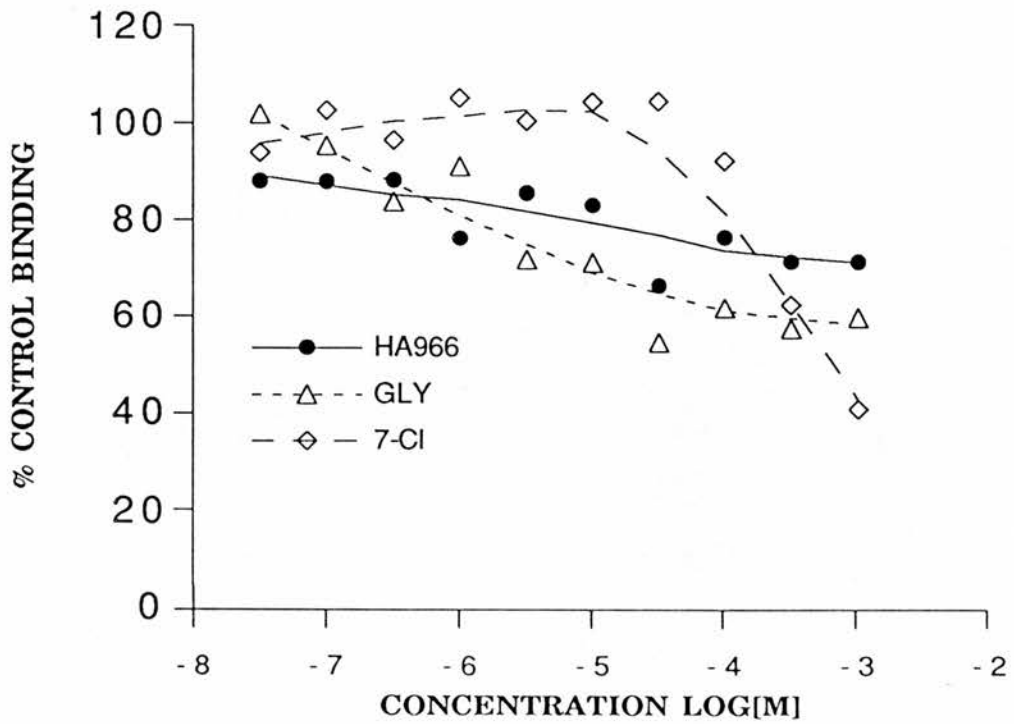


FIGURE 23: The effect of glycine ,HA-966 and 7-Clkyn on the binding of [^{3}H]D-AP5
Binding was measured as previously described in Fig.21 but in the presence of increasing concentrations of glycine (GLY), HA-966(HA966) or 7-Clkyn (7-Cl), (0.03 - 1000μM). Specific binding in the presence of inhibitor was expressed as a percentage of binding under control conditions. Raw data were fitted by least squares to the logistic expression: $Y = MX^P/(X^P + IC_{50})$, K_d and Bmax values were estimated. n = 1 for glycine and 7-Clkyn although the experiment was repeated with similar results. n = 3 for HA-966

[^{3}H]CPP binding

7-Clkyn had a different effect on [^{3}H]CPP binding from both glycine and HA-966. Increasing concentrations of 7-Clkyn inhibited [^{3}H]CPP binding, in a concentration dependent manner, although the inhibition was incomplete (Fig.22). In the presence of 1mM 7-Clkyn 28 ± 4.8% of [^{3}H]CPP was not inhibited. The IC_{50} value for this inhibition was calculated as 104.1 ± 16.3μM (n = 3).

[^{3}H]D-AP5 binding

7-Clkyn unlike HA-966 and glycine had a very similar effect on [3H]D-AP5 binding as the effect already reported for [^{3}H]CPP. 7-Clkyn inhibited the binding in a concentration dependent manner. Around 40% of binding remained insensitive to inhibition even by 1mM 7-Clkyn (Fig.23). The IC_{50} for this inhibition is approximately 100μM, similar to that calculated for inhibition of [^{3}H]CPP binding.

3.4.7 The effect of glycine on the inhibition of [^{3}H]CPP binding

One proposal of the actions of glycine is that it may promote the "agonist" state of the NMDA receptor (Monaghan *et al*, 1988). The effect of 3μM glycine, a concentration producing maximal stimulation of [^{3}H]CPP binding, was investigated on the inhibition of [^{3}H]CPP binding by CPP (Fig.24A), L-glutamate (Fig.24B), D-AP5 (Fig.24C) and 7-Clkyn (Fig.24D).

Glycine (3μM) had no significant effect on the inhibition of [^{3}H]CPP binding by L-glutamate and CPP (Table 8; Fig.24A and 24B). The K_d and B_{max} values were not significantly different from binding in the absence of glycine. The IC_{50} for D-AP5 inhibition was however affected, with it becoming less effective as an inhibitor of [^{3}H]CPP binding ($P < 0.05$; Table 8). Glycine had no significant effect on inhibition by 7-Clkyn. 27.4 ± 6.5% of binding still remained in the presence of 1mM 7-Clkyn and 3μM glycine. This is not significantly different from in the absence of glycine (Section 3.5.6).

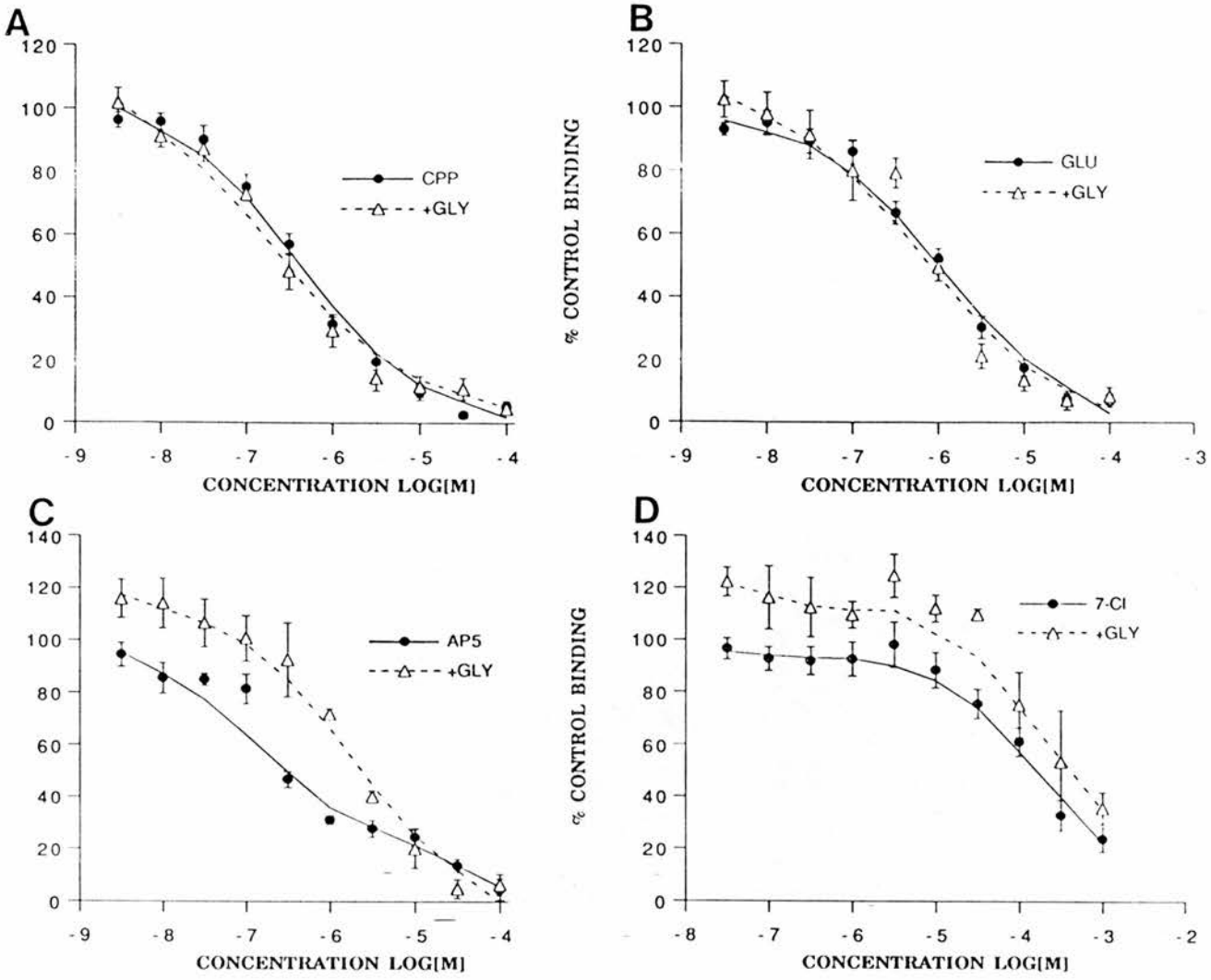


FIGURE 24: The effect of glycine on the inhibition of [^{3}H]CPP binding
[^{3}H]CPP binding was measured as described in Fig. 20, but in the presence of glycine (+GLY; 3μM) and increasing concentrations of (A), CPP (n = 5): (B), L-glutamate (n = 3; GLU): (C), D-AP5 (n = 3; AP5) and (D), 7-Clkyn (n = 3; 7-Cl), (0.03 - 1000μM). Specific binding was calculated and expressed as a percentage of binding under control conditions. Data were fitted by least squares to the logistic expression: $Y = MX^p/(X^p + IC_{50})$, K_d, K_i and Bmax values were estimated.
Each value represents mean ± sem.

In the presence of low concentrations of D-AP5 and 7-Clkyn, glycine increased binding to around 120% of control values. This was not seen with CPP or L-glutamate.

3.4.8 The effect of HA-966 on the inhibition of [^{3}H]CPP binding

The effect of 100μM HA-966 was examined on the inhibition of [^{3}H]CPP binding by CPP (Fig.25A), L-glutamate (Fig.25B), D-AP5 (Fig.25C) and 7-Clkyn (Fig.25D). Concentration dependent inhibition by each of these four compounds was still seen in the presence of HA-966 (100μM). HA-966 significantly increased the affinity of [^{3}H]CPP for its binding site resulting in a K_d of 0.21 ± 0.04μM (Table 8; $P < 0.05$). The B_{max} was reduced although not significantly.

The affinity of L-glutamate for the [^{3}H]CPP binding site was significantly decreased compared to control conditions in the presence of HA-966. The affinity of D-AP5 was also decreased compared to control (Table 8).

The IC_{50} for inhibition by 7-Clkyn was significantly decreased in the presence of HA-966 compared to control conditions, showing an increase in affinity for the [^{3}H]CPP binding site ($p < 0.05$; Table 8). 32.6 ± 4.4% of binding remained in the presence of 1mM 7-Clkyn and 100μM HA-966.

3.4.9 The effect of HA-966 and glycine on [^{3}H]CPP binding

1μM HA-966 did not have a significant effect on [^{3}H]CPP binding compared to control, and did not effect [^{3}H]CPP binding in the presence of increasing concentration of glycine (Fig.26). The weak stimulatory and inhibitory effects were still apparent. 100μM HA-966 resulted in 200% of control [^{3}H]CPP binding at low glycine concentrations. However as the glycine concentration increased the proportion of binding increased by HA-966 was inhibited to control levels of binding. The IC_{50} for this inhibition was 6.6 ± 1.5μM.

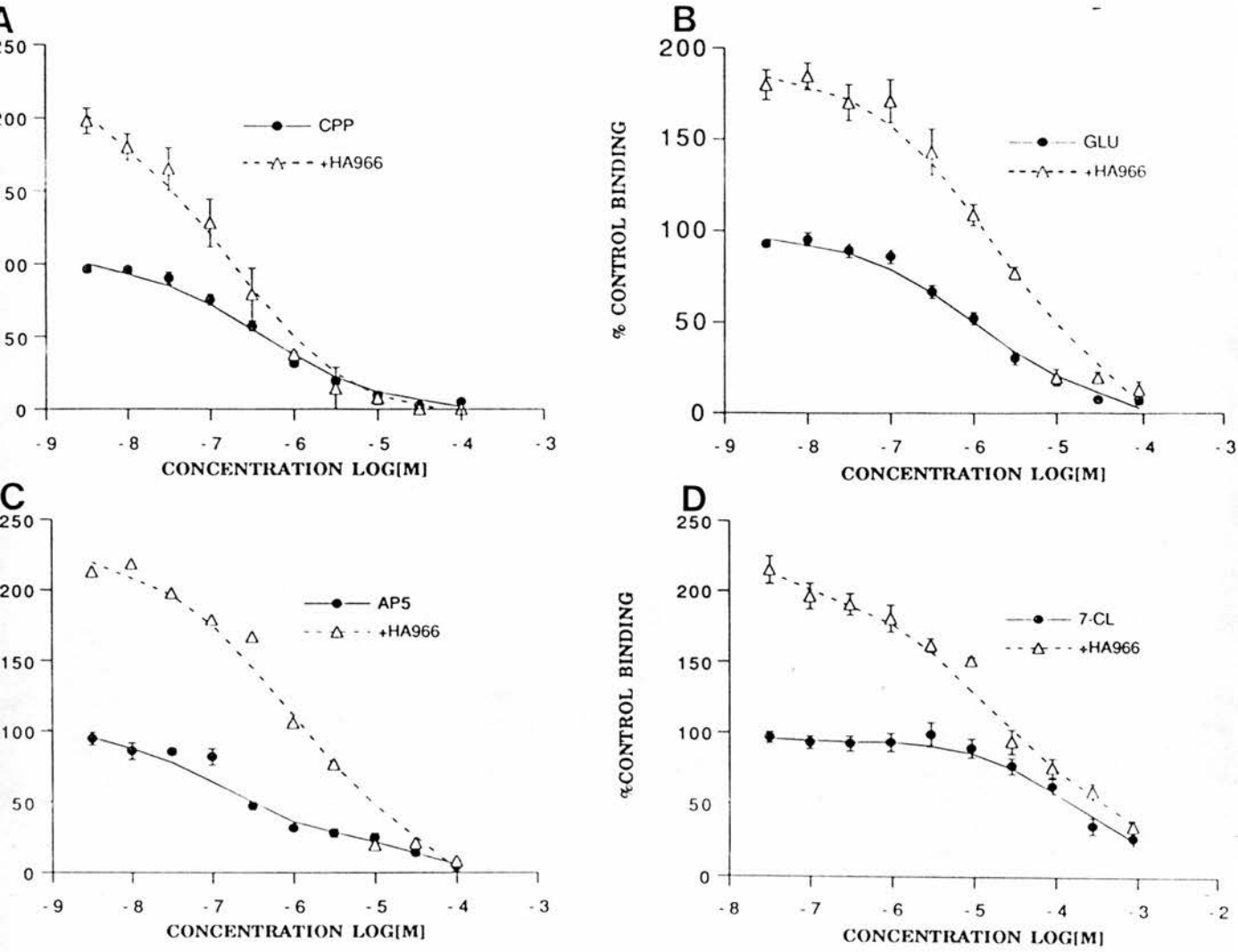


FIGURE 25; The effect of HA-966 on the inhibition of [^{3}H]CPP binding
Binding of [^{3}H]CPP was carried out as described in Fig.20 but in the presence of HA-966(+HA966:100μM) and increasing concentrations of (A),CPP (n = 3), (B), L-glutamate (n = 3; GLU); (C), D-AP5 (n = 1; AP5) and (D), 7-Clkyn(n = 3; 7-Cl), (0.03 - 1000μM). Specific binding was calculated and expressed as a percentage of binding under control conditions. Data were fitted by least squares to the logistic expression: $Y = MX^P / (X^P + IC_{50})$, K_d, K_i and Bmax values were estimated.
Each value represents the mean ± sem.

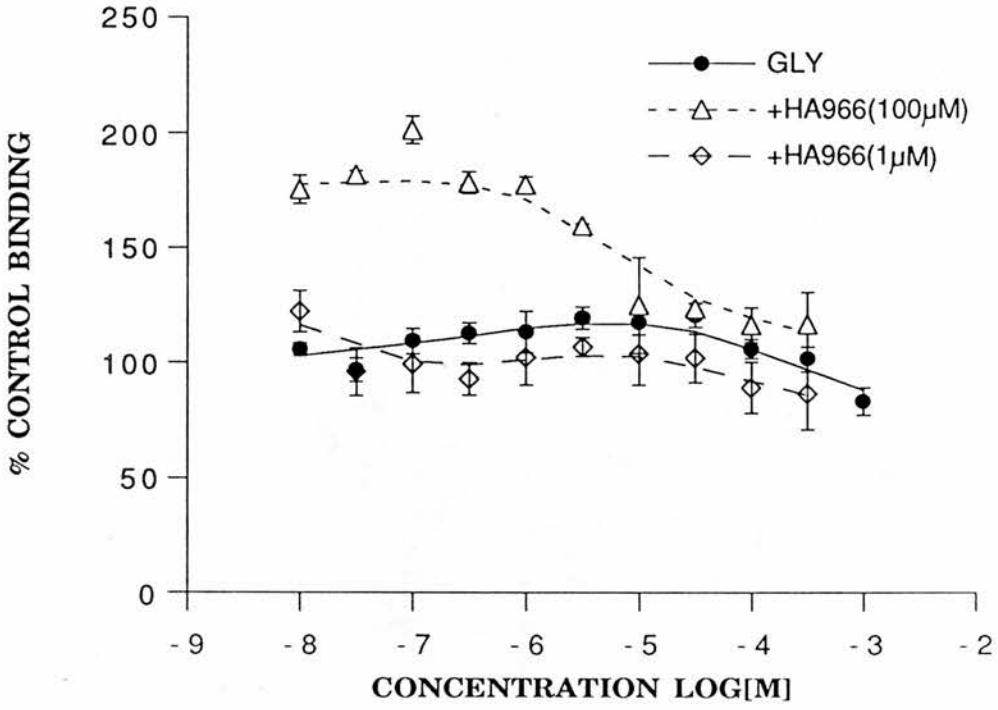


FIGURE 26; The effect of HA-966 and glycine on the binding of [^{3}H]CPP
Binding of [^{3}H]CPP was carried out as described in Fig.20 but in the presence of HA-966 (+HA966 1 or 100μM) and increasing concentrations of glycine (gly), (0.01-1000μM). Specific binding was calculated and expressed as a percentage of binding under control conditions. Data were fitted by least squares to the logistic expression: $Y = MX^P / (X^P + IC_{50})$, K_i values were estimated.
Each value is the mean ± sem of 3-5 individual experiments.

3.4.10 The effect of glycine and 7-Clkyn on [^{3}H]CPP binding

The effect of 1mM 7-Clkyn was tested on the binding of [^{3}H]CPP in the presence of increasing concentrations of glycine (Fig.27A). Binding was reduced to approximately 20% of control levels at all concentrations of glycine tested (0.01 - 1000μM). This is equivalent to binding in the presence of 7-Clkyn alone. Thus glycine does not modulate the binding of [^{3}H]CPP which remains in the presence of 1mM 7-Clkyn.

3.4.11 The effect of HA-966 and 7-Clkyn on [^{3}H]CPP binding

7-Clkyn (1mM) reduced [^{3}H]CPP binding to around 30% of control levels. Increasing concentrations of HA-966 led to complete inhibition of this component of binding which was normally insensitive to 7-Clkyn (1mM). An IC_{50} of 1.4μM was calculated for this effect (Fig.27B).

3.5 AN INVESTIGATION INTO THE ONTOGENY OF THE NMDA RECEPTOR

3.5.1 Introduction

The objective of the study was to investigate the ontogeny of the NMDA receptor in the rat CNS. This was carried out by measuring the binding of [^{3}H]CPP and [^{3}H]dizocilpine to the neurotransmitter site and the ion channel site on the NMDA receptor complex respectively. Membranes were prepared from rats of postnatal ages between PND0-PND28 and also from rats at PND90. PND90 in this study has been classed as adult and is used as a comparison to data obtained between PND0 and PND28. [^{3}H]Dizocilpine binding was measured using both whole membranes (previously frozen) and synaptosomal membranes (fresh). [^{3}H]CPP binding was measured using only a synaptosomal membranes preparation since binding to whole membranes was unsuccessful (Section 3.2). Specific binding at a single ligand concentration was measured for [^{3}H]CPP and [^{3}H]dizocilpine. Binding of [^{3}H]dizocilpine was also measured in the presence of L-glutamate and/or glycine to

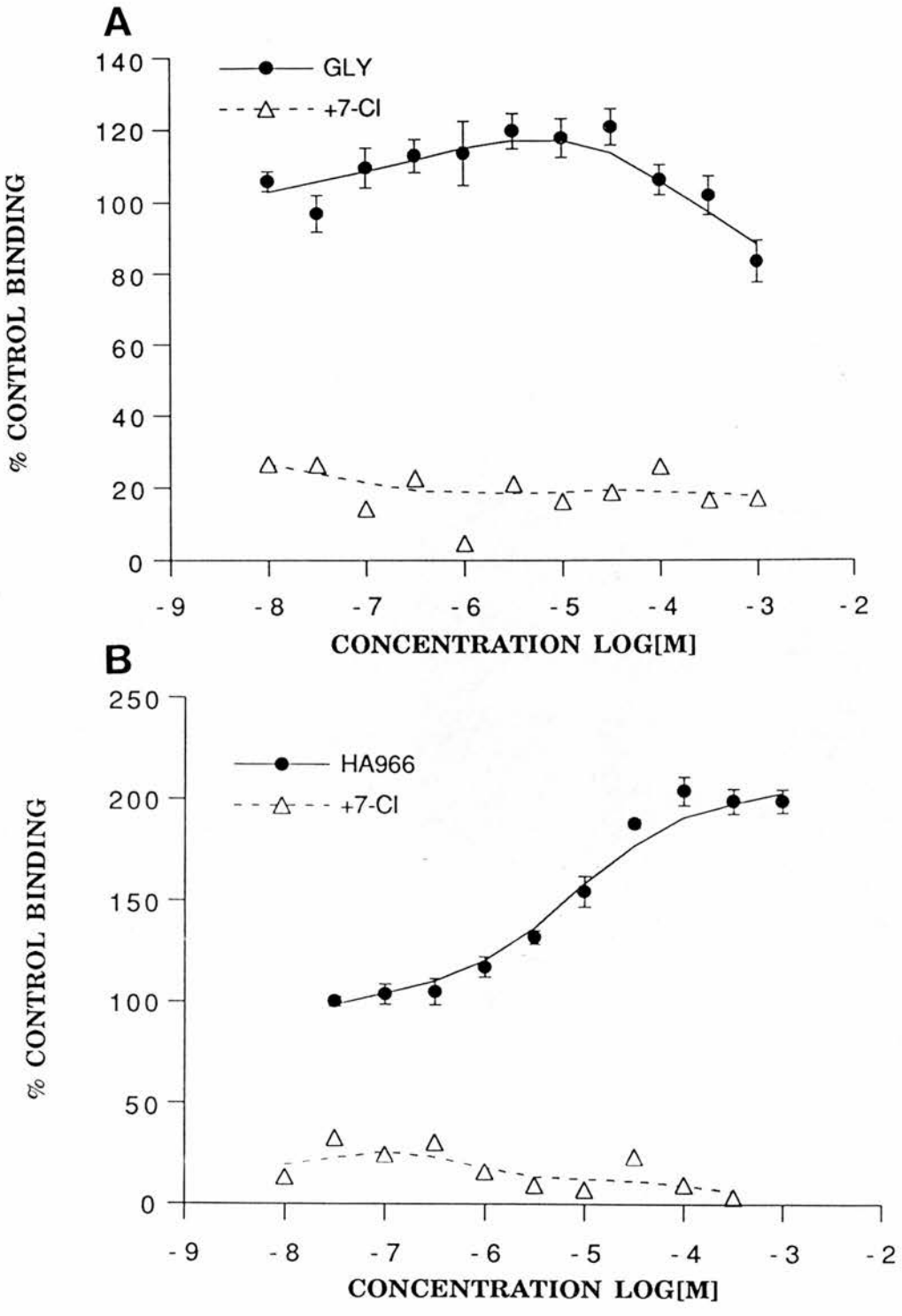


FIGURE 27: The effect of glycine ,HA-966 and 7-Clkyn on the binding of [^{3}H]CPP
Binding was measured as previously described in Fig.20 but in the presence of increasing concentrations of glycine (A; GLY) or HA-966(B; HA966),(0.03-1000μM) in the presence of 7-Clkyn (+7-Cl;1mM). Specific binding in the presence of inhibitor was expressed as a percentage of binding under control conditions. Raw data were fitted by least squares to the logistic expression: $Y = MX^P/(X^P + IC_{50})$, K_d and Bmax values were estimated. n = 1 for glycine and 7-Clkyn and n = 3 for HA-966.

investigate underlying regulatory mechanisms which may alter during postnatal development. Binding data were expressed both per mg protein and per mg wet weight tissue, since the protein content of the brain alters postnatally. The alteration in protein content may have some bearing on the resultant postnatal profile of binding. Using both plots, binding can be compared and the influence of protein alteratins assessed. The alteration in postnatal protein content of the membranes used in this study is shown in Fig.28 for whole and synaptosomal membranes. Whole membranes have the higher protein content throughout postnatal development.

Specific binding at one ligand concentration is not an ideal method of expressing binding data. Therefore K_d and B_{max} values were calculated for both ligands thus giving an indication of receptor affinity and receptor number. This was not always possible however when at young ages, (e.g. at ages less than PND8) dose-dependent inhibition and modulation could not be reliably measured. In such circumstances specific binding at a single ligand concentration was the only measurement available for comparisons between ages, membranes or experimental conditions. The effects of L-glutamate and glycine were investigated on the binding of [^{3}H]dizocilpine, at different postnatal ages. Calculation of EC_{50} values provided a quantitative parameter describing their ability to modulate binding.

Fig.29A shows an example of data for [^{3}H]dizocilpine binding throughout postnatal development. Although the increase in binding with postnatal age can be seen it is difficult to compare data between ages since at some ages only one measurement was made. Figure 29B shows the alternative method of presenting this data which allows statistical analysis to be carried out. The data is grouped at four day age intervals and is presented in the form of a histogram. One way analysis of variance can be applied to the data. Ages where binding is significantly different from that at PND90 are indicated. All further binding data is therefore presented in this format. The postnatal development of [^{3}H]dizocilpine and [^{3}H]CPP binding, at a single ligand concentration, to the NMDA receptor neurotransmitter site and associated ion channel was measured in membranes prepared from pooled cortices and hippocampi as

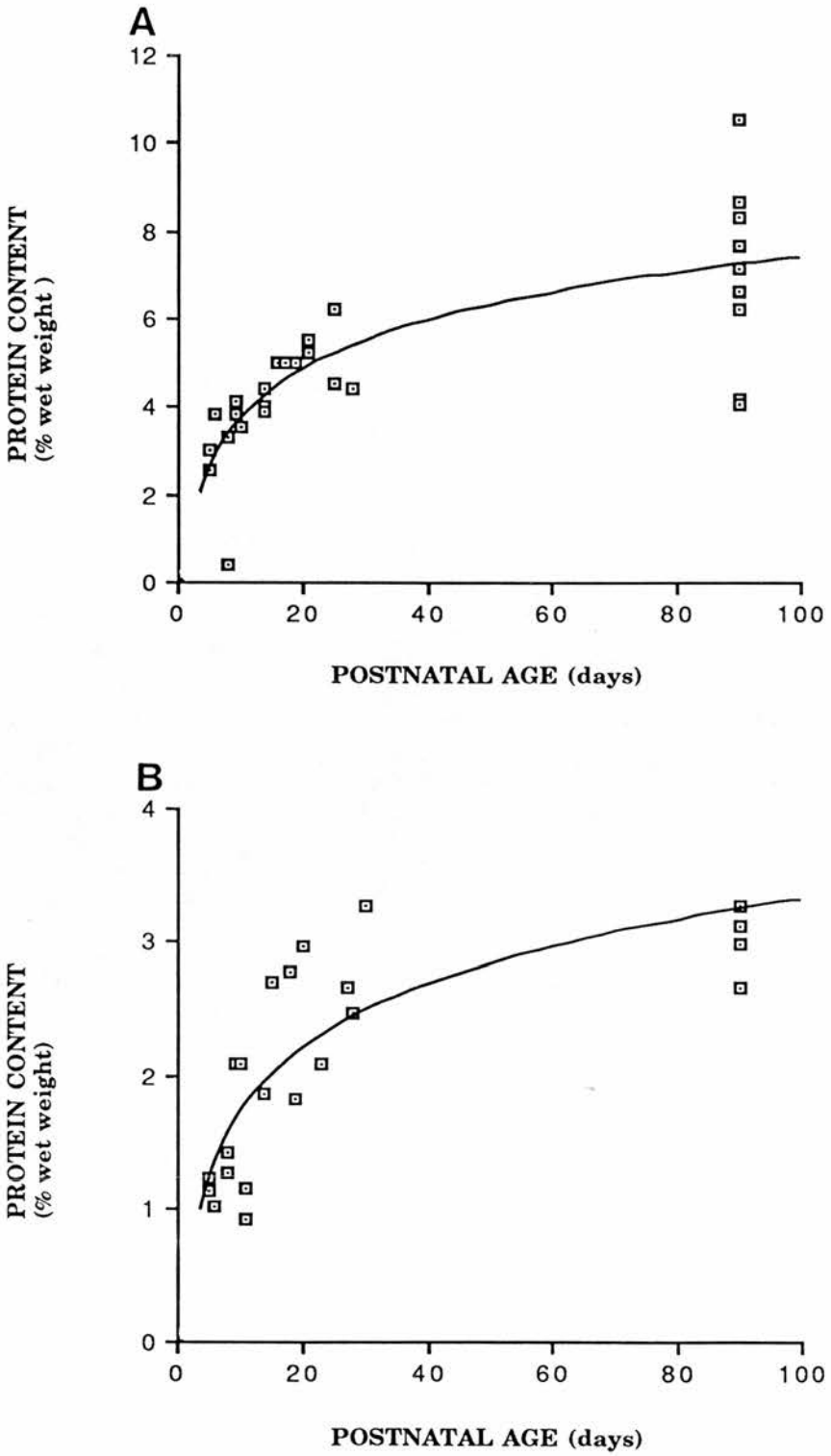


FIGURE 28: Protein content of membrane samples used in postnatal binding studies
The protein content of membrane samples was measured using the method of Bradford(1978). An increase in protein concentration was seen with increasing age in both whole(A) and synaptosomal(B) membranes. Whole membranes have the higher protein content at all ages tested. A smooth curve was fitted to the data showing an increase in protein content with increasing age.

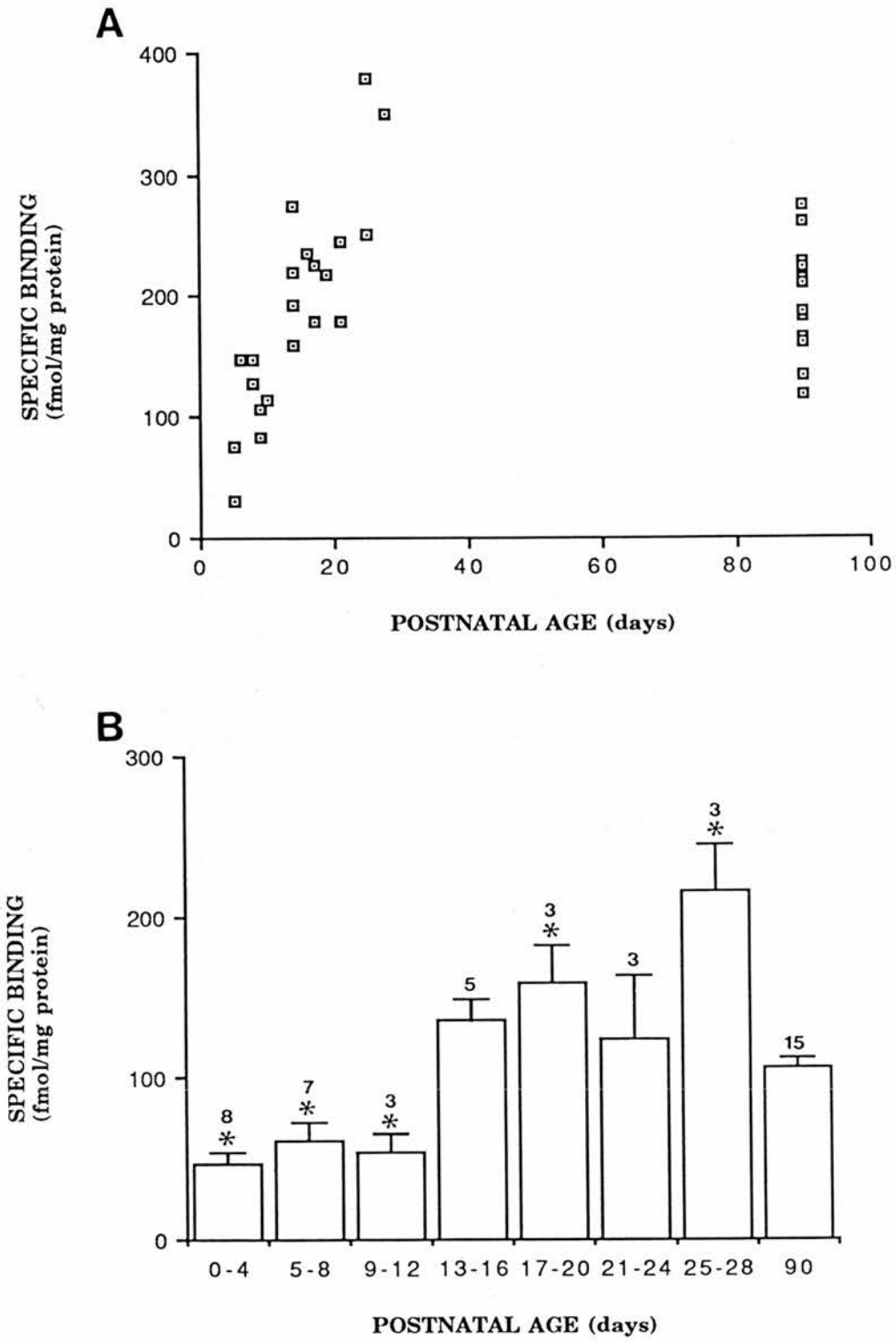


FIGURE 29: Comparison of a plot of all data points and a plot of grouped data for postnatal binding

Whole membranes were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) in the presence or absence of dizocilpine (30μM), to measure total and non-specific binding. Binding was measured in the presence of L-glutamate (10μM). Specific binding was calculated by subtracting non-specific binding from total binding and expressed per mg protein. Individual points were plotted against age(A) or grouped at four day age intervals (B). One-way analysis of variance was applied to (B). This revealed a significant difference between binding at different postnatal ages.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

described previously. The data shall be presented as follows; (i) [^{3}H]Dizocilpine binding to whole membranes (Section 3.7), (ii) [^{3}H]Dizocilpine binding to synaptosomal membranes (Section 3.8) and (iii) [^{3}H]CPP binding to synaptosomal membranes (Section 3.9).

3.5.2 Brain and body development

Brain tissue and body weights were recorded during postnatal development in Wistar Cob rat pups (PND0 - PND28). No distinction was made between males and females. Body weights increase gradually until PND28 but increase at a more rapid rate after 3 weeks of age (Fig.30A). Tissue weight as illustrated in Figure 30B represents the combined weight of cerebral cortices and hippocampi (wet weight) as used for membrane preparations. In contrast to whole body weight, tissue weight increases rapidly until PND17-20. After this age growth is much slower, as it approaches adult weight. Brain weight is therefore approaching adult levels at an earlier age than body weight. However, this is no reflection of the postnatal development of brain constituents.

3.6 [^{3}H]DIZOCILPINE BINDING TO WHOLE MEMBRANES

3.6.1 Control Conditions

Specific binding of [^{3}H]dizocilpine at a single ligand concentration was measured at various postnatal ages in the absence of L-glutamate and glycine (Fig.31). Specific binding was detected from PND0 and at all ages tested throughout postnatal development. Binding was lowest during the age range PND0-4 (Fig.31A; mean age 1.2d; 46.02 ± 7.44 fmol/mg protein; n = 8) and was less than half the amount of specific binding detected in adult tissue (PND90; 106.5 ± 6.01fmol/mg protein; n = 15). Binding remained low until PND9-12 after which it increased to levels higher than at PND90. Maximum binding was reached at PND25-28 (mean age 26.0d; 215.1 ± 29.6fmol/mg protein; n = 3). Binding at PND25-28 was approximately 200% of the PND90 level. Analysis of variance revealed a significant difference between binding

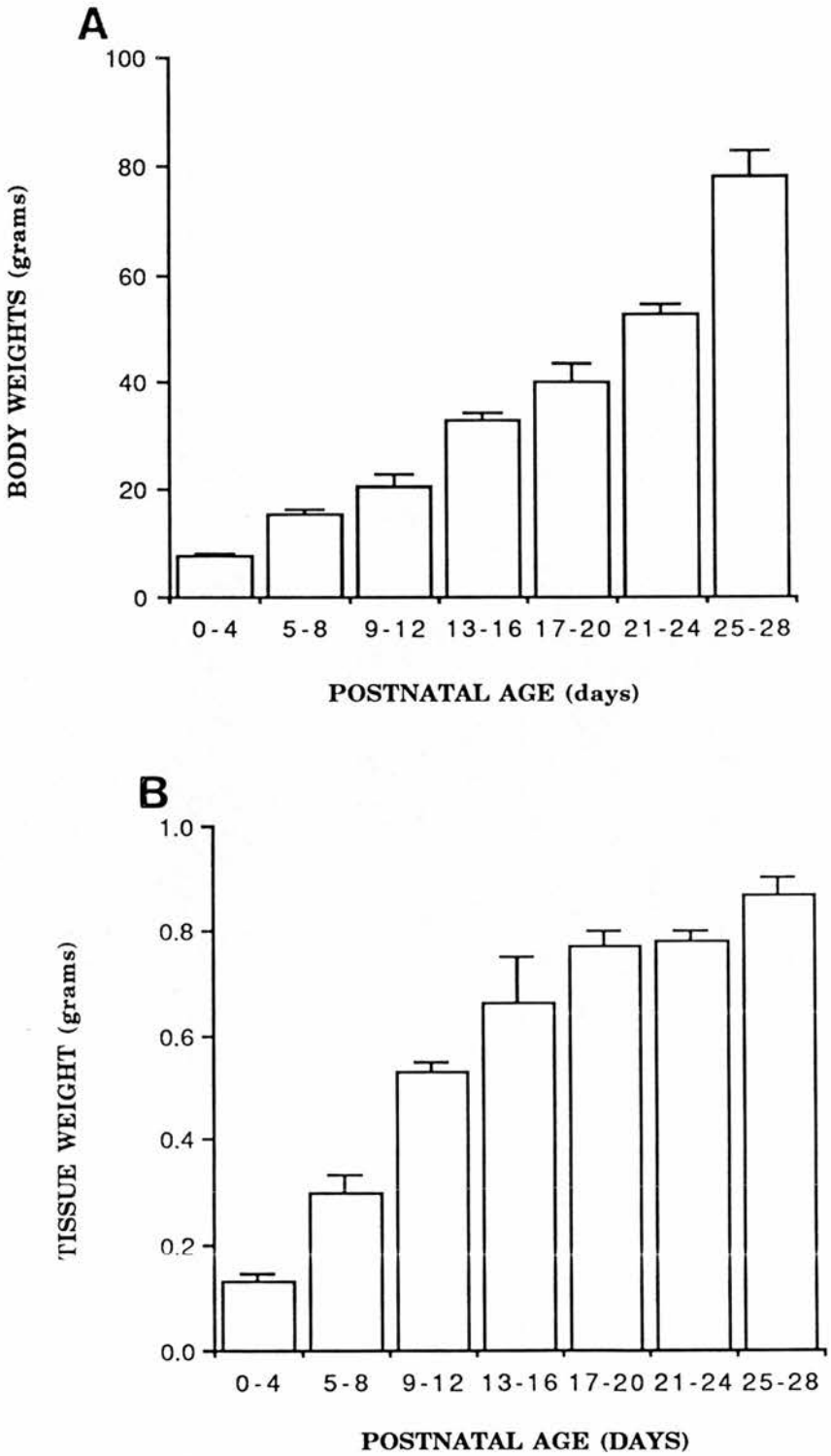


FIGURE 30: Rat brain and tissue weights during postnatal development
The increase in rat body weight (A) is slower than that of brain tissue weight (B) during the first four weeks of life. Tissue weight plateaus by PND25-28 while body weight is still increasing. Brain tissue weight is defined here as the tissue wet weight of pooled cortical and hippocampal tissue. Values are mean ± sem of 4 independent observations.

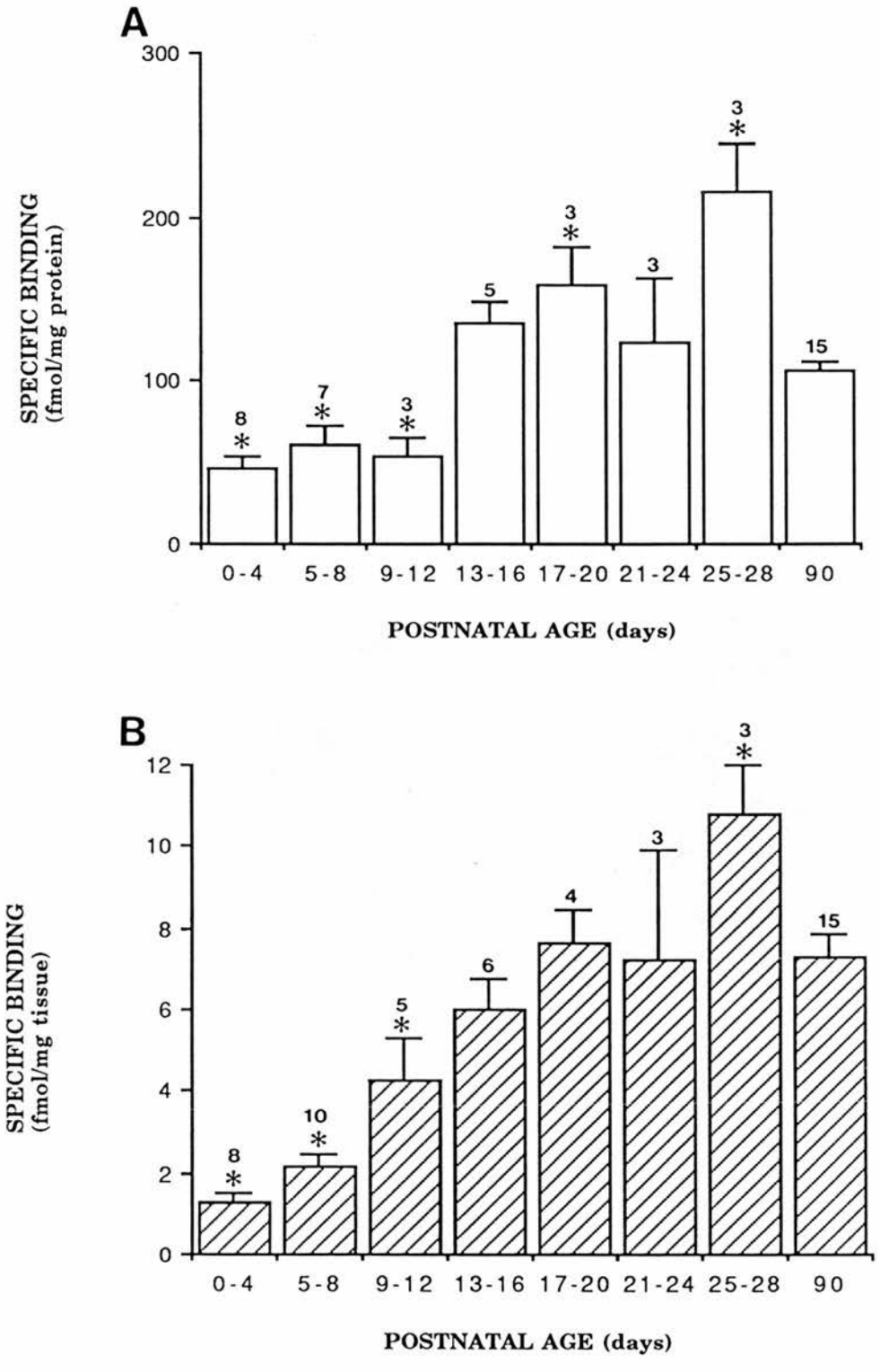


FIGURE 31: Postnatal binding of [^{3}H]dizocilpine to whole membranes
Membranes (previously frozen) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) in the absence or presence of dizocilpine (30μM) to measure total and non-specific binding. Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages ($p < 0.05$), see text for details.
*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

at different ages; $F(7) = 14.385$, $p < 0.01$. Further analysis using a t-test showed that until PND9-12 [^{3}H]dizocilpine binding was significantly lower than at PND90 ($p < 0.05$). At PND17-20 and PND25-28 binding was significantly higher than at PND90 ($p < 0.05$). At all other ages binding was not significantly different from at PND90.

When the data were expressed per mg tissue a different profile was seen. Binding increased gradually with increasing age (Fig.31B) unlike the sudden increase seen after PND9-12 when expressed per mg protein. Lowest specific binding was observed at PND0-4 (mean age 1.2d; 1.37 ± 0.19 fmol/mg tissue; n = 8). This accounts for a much smaller proportion (18%) of adult binding (PND90; 7.38 ± 0.45 fmol/mg tissue; n = 15) compared to when the data were expressed per mg of protein. Binding increased gradually reaching a maximum level at PND25-28 (mean age 26.0d; 10.74 ± 1.2fmol/mg tissue; n = 3). This level of binding was almost 150% of the PND90 value. A significant difference between binding at different postnatal ages was revealed with analysis of variance, $F(7) = 10.773$, $p < 0.01$. Further analysis revealed that binding between PND0 and PND12 was significantly lower than at PND90, while at PND25-28 it was significantly higher ($p < 0.05$). Binding at all other ages was not significantly different from binding at PND90.

3.6.2 Effect of L-glutamate on specific binding of [^{3}H]dizocilpine

L-glutamate modulation resulted in an increase in the specific binding of [^{3}H]dizocilpine compared to control binding (Section 3.3). This effect was measured throughout postnatal development from PND5-8 onwards (Fig.32).

The binding profile (Fig.32A) for postnatal binding was almost identical to that observed in the absence of L-glutamate. No increase in specific binding compared to control conditions could be measured at PND0-4. Binding remained low until PND9-12 thereafter increasing to levels higher than measured at PND90 (196.9 ± 11.8fmol/mg protein; n = 13). Maximal binding was seen at PND25-28 (mean age 26.0d; 326 ± 39.2fmol/mg protein; n = 3). Analysis of variance revealed a significant difference between binding at different postnatal ages, $F(7) = 18.898$, $p < 0.01$. Binding at ages

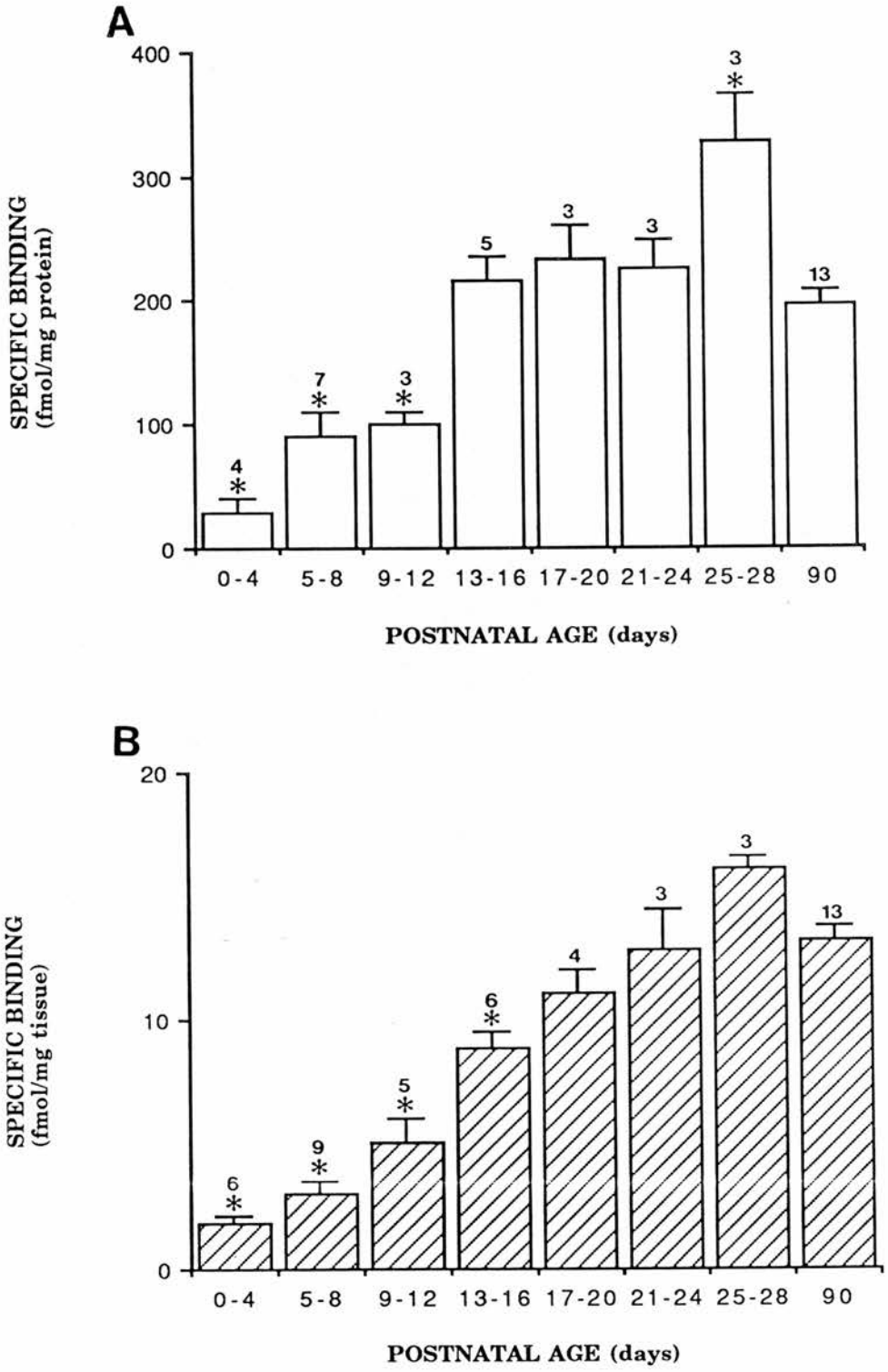


FIGURE 32: The effect of L-glutamate on the postnatal binding of [^{3}H]dizocilpine to whole membranes

Membranes(previously frozen) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) and L-glutamate (10μM). Dizocilpine (30μM) was used to measure non-specific binding. Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages ($p < 0.05$), see text for details.

*, $P<0.05$ when compared to PND90 value. Number of observations are indicated above each column.

between PND0-12 was significantly lower and binding at PND25-28 was significantly higher than that at PND90 ($p < 0.05$). Further analysis revealed that levels of binding not significantly different from PND90 occurred between PND13-24.

Data expressed per mg tissue (Fig.32B) again show a gradual increase in binding postnatally. Lowest binding was seen at PND0-4 (mean age 1d; 2.6 ± 0.19 fmol/mg tissue; $n = 6$) rising to peak binding levels at PND25-28 (mean age 26d; 16.09 ± 0.56 fmol/mg tissue; $n = 3$), representing around 20% and 120% of PND90 levels (13.39 ± 0.6 fmol/mg tissue; $n = 13$) respectively. A significant increase in binding was detected at PND0-4 compared to control conditions ($p < 0.05$). Significant differences between binding at different ages were revealed by analysis of variance, $F(7) = 33.409$, $p < 0.01$. Further analysis revealed that binding between PND0 and PND16 was significantly less than at PND90 ($p < 0.05$).

3.6.3 Extent of L-glutamate modulation

The extent to which [^{3}H]dizocilpine specific binding was enhanced by L-glutamate (10μM) during postnatal development is illustrated in Fig.33. At PND0-4, when expressed per mg protein (Fig.33A) L-glutamate appeared to reduce binding compared to control. From PND5 binding is increased at all ages compared to control conditions, to a similar extent. When expressed per mg tissue (Fig.33B) binding is enhanced at all ages to a similar extent. Analysis of variance was applied to both sets of data. When expressed per mg protein (Fig.33A), $F(7) = 2.478$, $p < 0.05$ a significant difference was found between ages. Further analysis revealed that the modulation at PND0-4 was significantly less than the modulation at all other ages ($p < 0.05$). The amount of binding was not significantly different from that seen under control conditions. No other differences were found. When expressed per mg tissue analysis of variance revealed no significant differences between ages (Fig.33B), $F(7) = 0.577$.

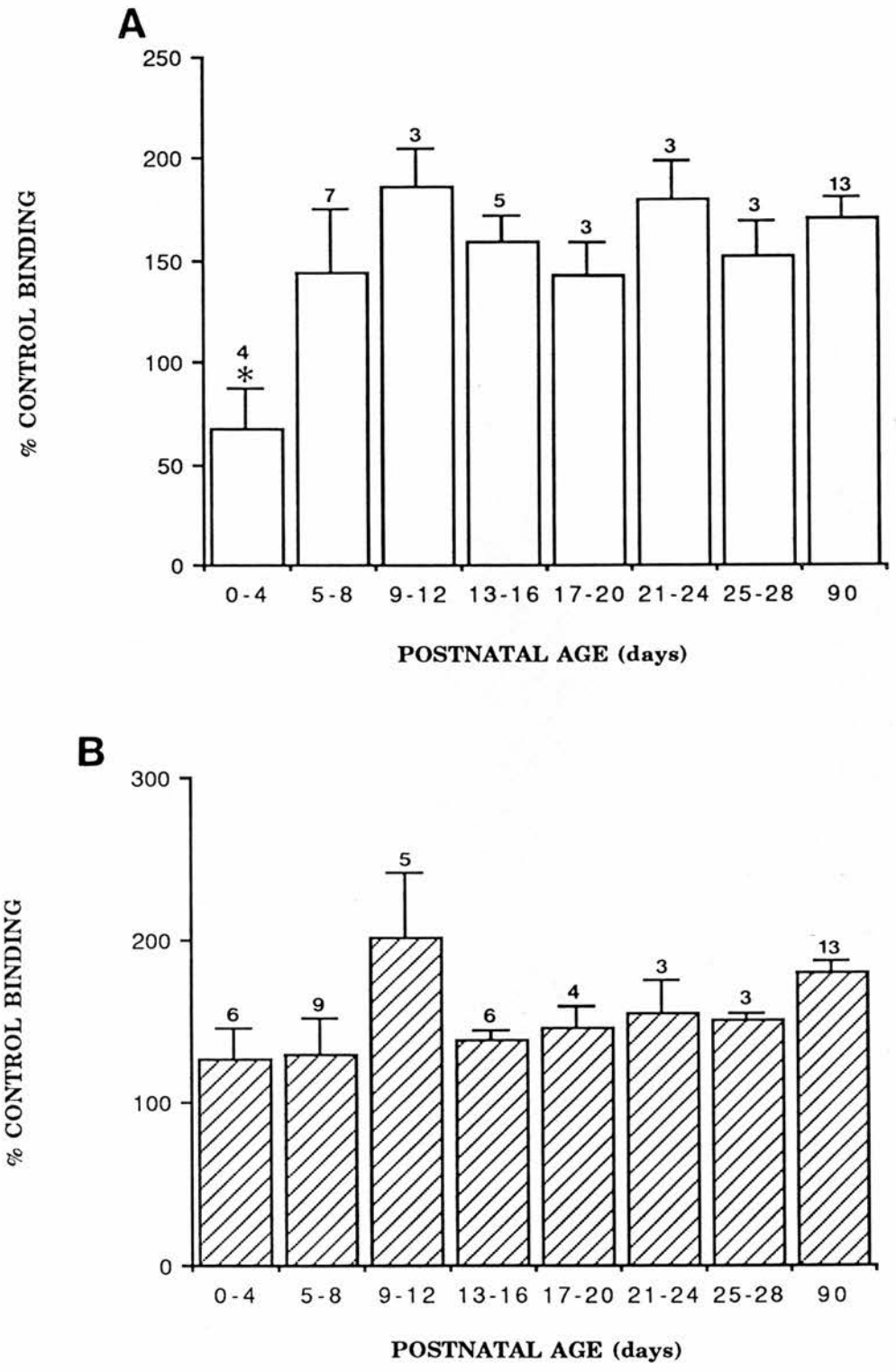


FIGURE 33: Extent of L-glutamate modulation of [^{3}H]dizocilpine binding to whole membranes

Each determination of binding in Fig.32 was expressed as a percentage of binding under control conditions both per mg protein (A) and per mg tissue (B), to evaluate whether the extent of modulation by L-glutamate altered during postnatal development. Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. A significant effect was seen in (A), ($p < 0.05$), see text for full details.

*, $P<0.05$ when compared to all other values. Number of observations are indicated above each column.

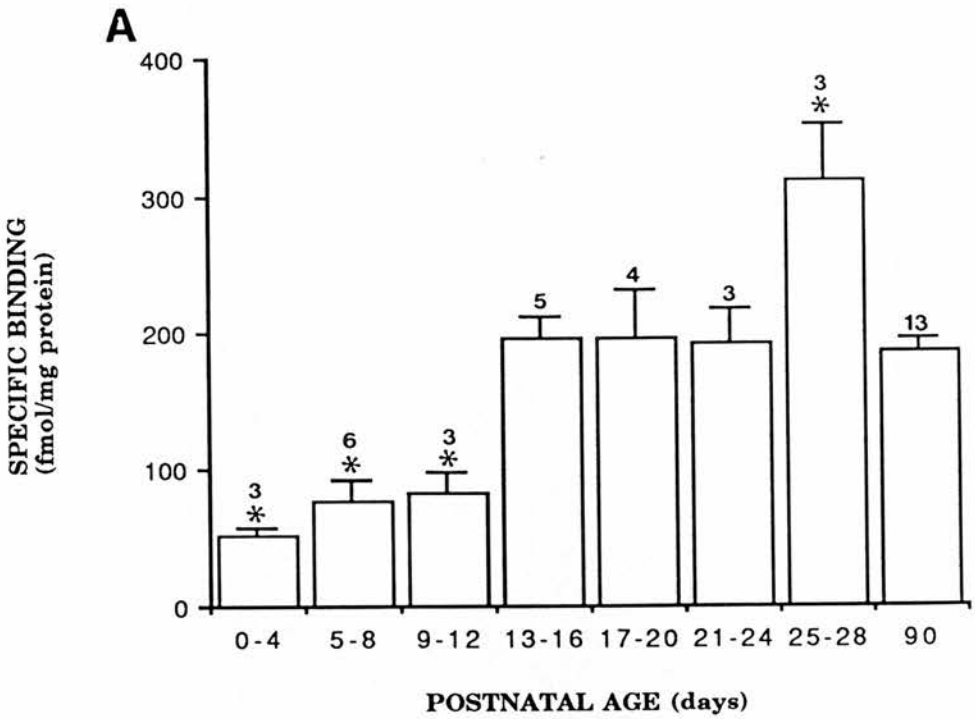
3.6.4 The effect of glycine on specific binding of [^{3}H]dizocilpine

The ontogenic binding profile for [^{3}H]dizocilpine binding during postnatal development in the presence of glycine (10μM), (Fig.34A), is almost identical to that seen in the presence of L-glutamate (Fig.32A). Binding was lowest at PND0-4 (mean age 1.7d; 51.95 ± 6.8 fmol/mg protein; n = 3) which unlike L-glutamate shows an increase compared to control conditions. Binding remained low until PND9-12 (mean age 9.3d; 84.2 ± 14.5 fmol/mg protein; n = 3) after which it increased. A peak level of binding was reached at PND25-28 (mean age 26.0d; 310.5 ± 4.1 fmol/mg protein; n = 3) declining thereafter to PND90 levels (185.8 ± 10.2 fmol.mg protein; n = 13). Analysis of variance revealed a significant difference between ages $F(7) = 15.597$, $p < 0.01$. Further analysis showed identical differences to those revealed in the presence of L-glutamate with binding between PND0 and PND12 being significantly lower than PND90 levels and binding at PND25-28 being significantly higher ($p < 0.05$).

When expressed per mg tissue (Fig.34B) the profile is also almost identical to that seen in the presence of L-glutamate (Fig.32B). At PND0-4 (mean age 1.3d; 2.9 ± 0.26fmol/mg tissue; n = 5) binding is lowest, being just over 20% of the adult value (13.27 ± 0.42 fmol/mg tissue; n = 13). Binding increases gradually with increasing age until a maximum level is reached, at PND25-28 (mean age 26.0d; 14.62 ± 0.45 fmol/mg tissue; n = 3). Analysis of variance revealed a significant difference between binding at different ages, $F(7) = 31.271$, $p < 0.01$. Binding between PND0-PND16 was significantly lower than that seen at PND90 ($p < 0.05$). Binding at all other ages was not significantly different to binding at PND90.

3.6.5 Extent of glycine modulation

The extent to which [^{3}H]dizocilpine binding was enhanced by glycine (10μM) during postnatal development is illustrated in Fig.35. Glycine modulation was seen at all ages. The extent of modulation did not vary at different postnatal ages regardless of the method of data expression. Analysis of variance was applied to both sets of data. $F(7) = 1.151$ and $F(7) = 1.039$, for data expressed per mg of protein and tissue



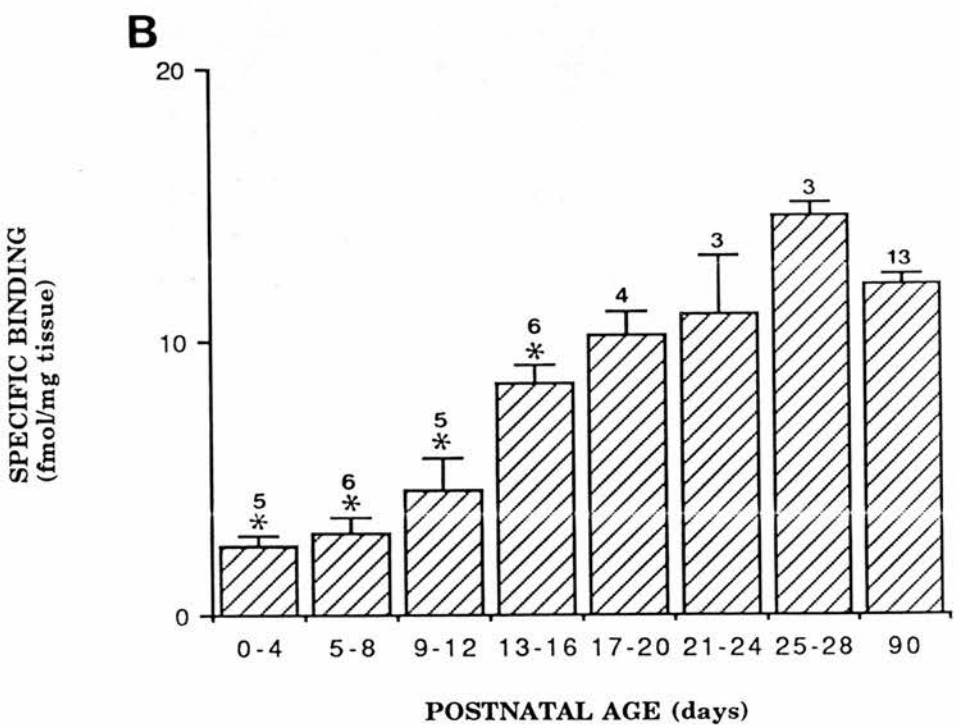


FIGURE 34: The effect of glycine on the postnatal binding of [^{3}H]dizocilpine to whole membranes

Membranes(previously frozen) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) and glycine (10μM). Dizocilpine (30μM) was used to measure non-specific binding. Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between ages ($p < 0.05$), see text for details.
*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

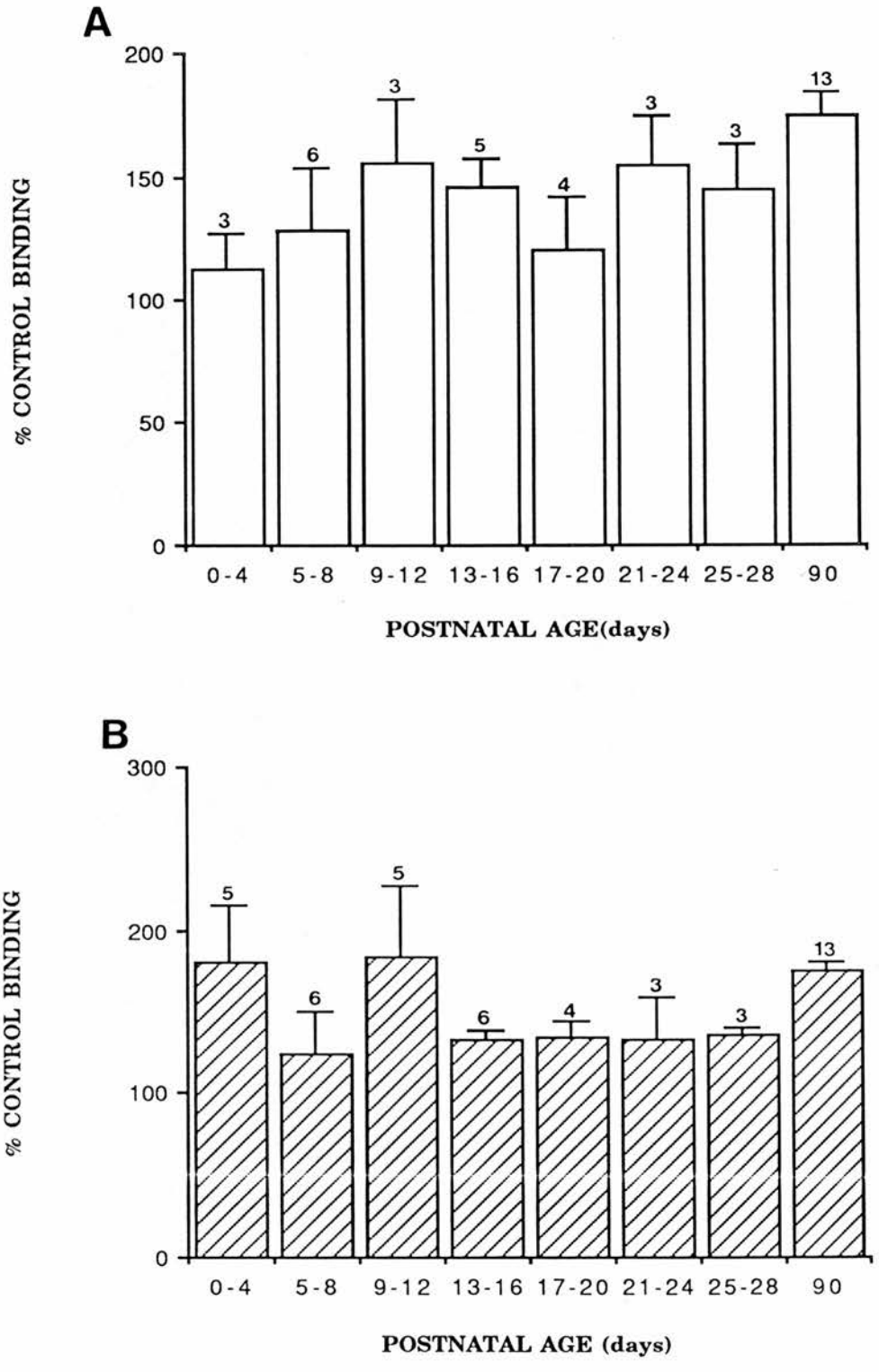


FIGURE 35: Extent of glycine modulation of [^{3}H]dizocilpine binding to whole membranes

Each determination of binding in Fig.34 was expressed as a percentage of binding under control conditions both per mg protein (A) and per mg tissue (B),to evaluate whether the extent of modulation by glycine altered during postnatal development. Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. No significant differences were seen between ages.
Number of observations are indicated above each column.

respectively. These very low F values indicated that no significant differences exist between ages and that the extent of modulation by glycine did not alter significantly with increasing age.

3.6.6 The effect of L-glutamate and glycine on [^{3}H]dizocilpine binding

As well as enhancing the binding of [^{3}H]dizocilpine when administered individually, these two amino acids increase binding when present in combination. The basic profiles gained for the individual amino acids (Figs.32 and 34) have paralleled the control profiles (Fig.31). Fig.36A shows that the developmental profile for [^{3}H]dizocilpine binding in the presence of both L-glutamate and glycine is also similar to these profiles. Binding at PND0-4 (Fig.36A; mean age 0.8d; 59.2 ± 11.4 fmol/mg protein; n = 6) was approximately one quarter of the final adult value (PND90; 224.9 ± 22.04 fmol/mg protein; n = 13). The greatest amount of binding was seen at PND25-28 (mean age 26.0d; 339.9 ± 47.7 fmol/mg protein; n = 3), when binding peaked at around 150% of the PND90 level. Analysis of variance revealed significant differences between binding at different ages in the presence of both amino acids which were equivalent to those seen with each individual amino acid, $F(7) = 10.333$, $p < 0.01$. Binding between PND0 and PND12 was significantly lower and binding at PND25-28 significantly higher than binding seen at PND90 ($p < 0.05$).

Expressing data per mg tissue (Fig.36B) resulted in a profile whereby binding increased to PND90 levels. Binding at PND0-4 (mean age 1.1d; 1.77 ± 0.16 fmol/mg tissue; n = 7) represents only 11% of PND90 binding (15.93 ± 0.7 fmol/mg tissue; n = 13). An F value of $F(7) = 6.478$, $p < 0.01$ was found when analysis of variance was applied to the data. Binding between PND0 and PND20 was significantly less than that at PND90. Adult levels of binding were reached at the age of PND21-24 (mean age 22.3d; 14.94 ± 0.35 fmol/mg tissue; n = 3).

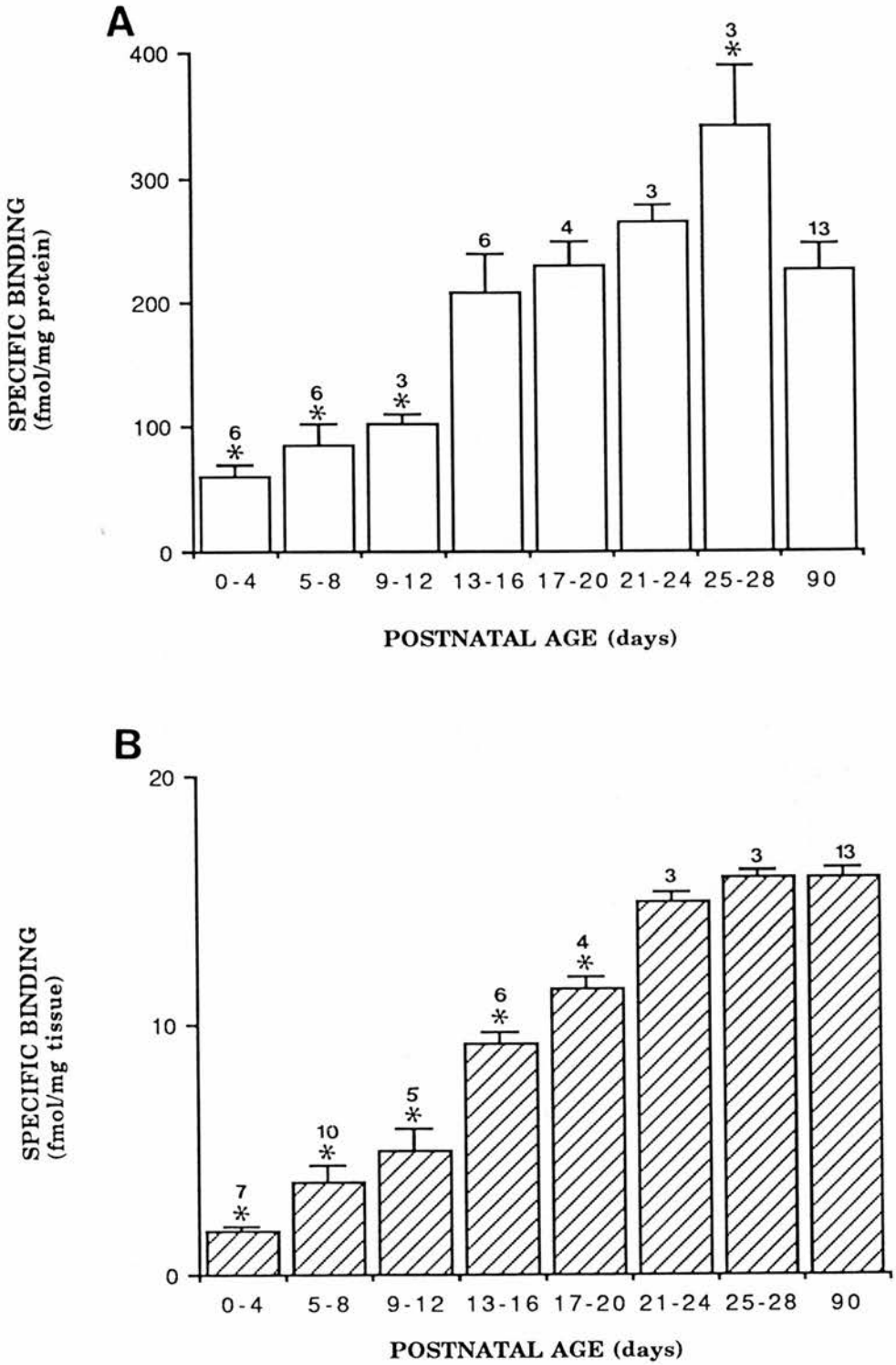


FIGURE 36: The effect of L-glutamate and glycine on the postnatal binding of [^{3}H]dizocilpine to whole membranes

Membranes(previously frozen) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) and L-glutamate and glycine (both 10μM). Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages ($p < 0.05$),see text for details.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

3.6.7 Extent of modulation by L-glutamate and glycine

The extent of modulation by L-glutamate and glycine is shown in Fig.37. When expressed per mg protein (Fig.37A), as with glycine, the extent of modulation does not vary postnatally. However, this is different to L-glutamate (Fig.33A) when at PND0-4 no modulation was observed. Analysis of variance revealed no significant differences between ages, $F(7) = 1.151$ when expressed per mg protein. When expressed per mg tissue a significant effect is found $F(7) = 2.328$; $p < 0.05$. Further analysis revealed that modulation at PND9-12 and PND21-24 was equivalent to that seen at PND90 while at all other ages the degree of modulation was significantly lower.

3.6.8 Comparison of specific binding between control and modulatory conditions

Binding at each age was compared with binding in each other experimental condition using two way analysis of variance to find out whether binding was significantly increased compared to control conditions by L-glutamate and/or glycine. A significant difference was found between conditions, $F(3) = 17.096$, $p < 0.01$ and $F(3) = 33.575$, $p < 0.01$ for data expressed per mg protein and per mg tissue respectively. Binding in the presence of L-glutamate and/or glycine was significantly greater than under control conditions ($p < 0.05$). Binding in the presence of glycine was significantly less than seen with L-glutamate and glycine ($p < 0.05$). A similar analysis was performed for all measurements of percentage modulation between treatments. No significant effect was found, $F(2) = 2.096$ and $F(2) = 0.577$ for data expressed per mg protein and mg tissue respectively. L-Glutamate, glycine and the combination of the two are therefore modulating binding of [^{3}H]dizocilpine to a similar extent.

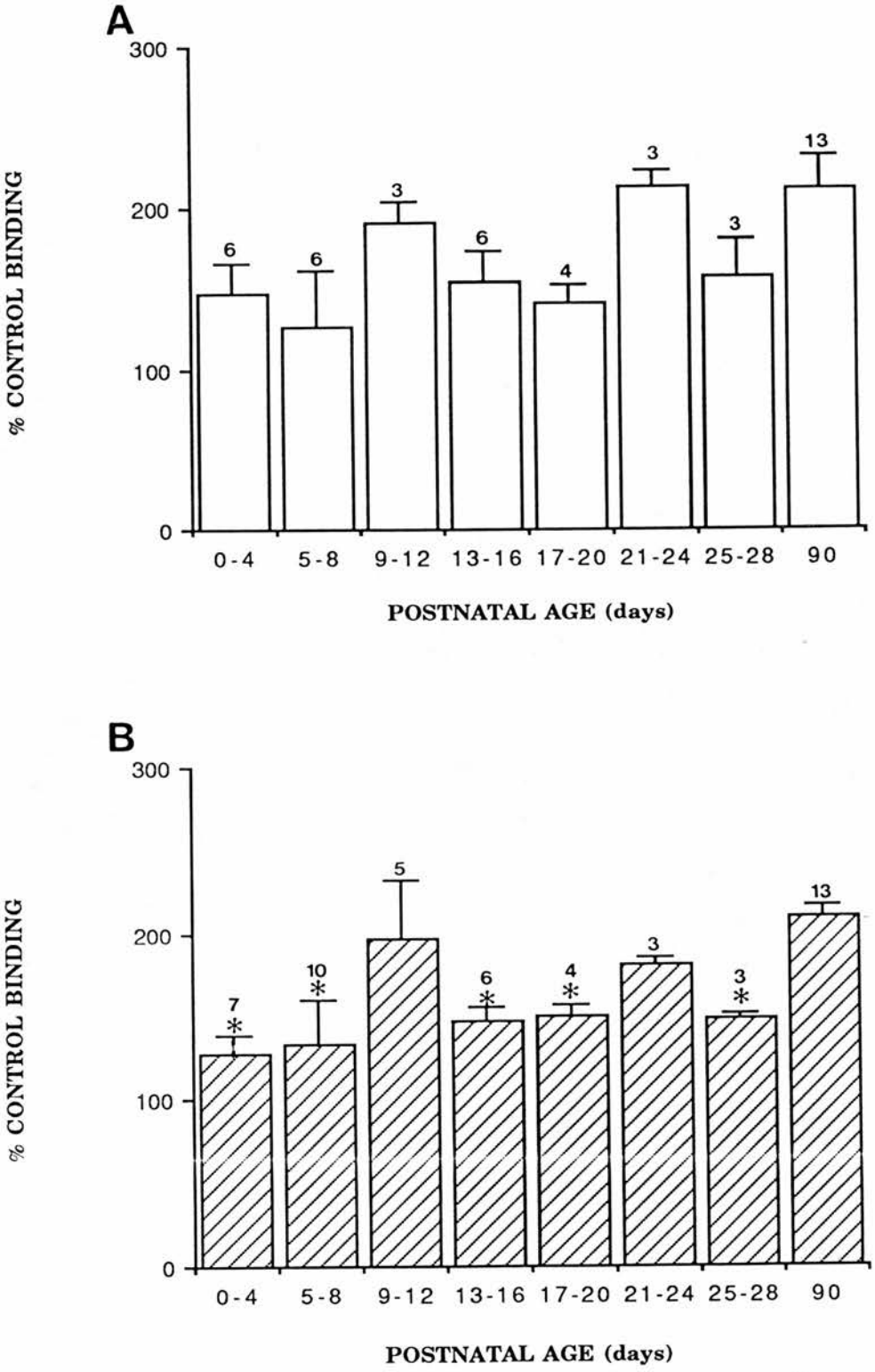


FIGURE 37: Extent of modulation of [^{3}H]dizocilpine binding to whole membranes by L-glutamate and glycine

Each determination of binding in Fig.36 was expressed as a percentage of binding under control conditions both per mg protein (A) and per mg tissue (B), to evaluate whether the extent of modulation by glycine altered during postnatal development. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages in (B), ($p < 0.05$), see text for details.
*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

3.6.9 Determination of the differences in specific binding during postnatal development

It is clear therefore that [^{3}H]dizocilpine binding to a whole membrane prepared from rat brain tissue varies in amount throughout postnatal development. This binding can be modulated by the amino acids L-glutamate and glycine. It is therefore important to try and find the underlying reasons for the changes in [^{3}H]dizocilpine binding. To this end K_d and B_{max} values under control and modulatory conditions have been measured. EC_{50} values were also measured for L-glutamate and glycine modulation of binding.

3.6.10 Determination of K_d values for [^{3}H]dizocilpine binding during postnatal development

Increasing concentrations of unlabelled dizocilpine were used to inhibit [^{3}H]dizocilpine in an attempt to measure the affinity of this compound for its binding site. Throughout postnatal development dizocilpine inhibited [^{3}H]dizocilpine binding to whole membranes in a dose-dependent manner in the absence and presence of amino acid modulators (Fig.38), as previously demonstrated in mature tissue. K_d values are displayed in Table 9A for binding throughout postnatal development under control conditions. Reliable and reproducible inhibition of [^{3}H]dizocilpine binding was not easily measured at PND0-4 because of the low specific to total binding ratio. Therefore it was not always possible to analyse data to obtain K_d and B_{max} values at this age. K_d values do not alter between ages during development as indicated by analysis of variance , $F(6) = 0.785$. Analysis of variance revealed that n_H does not change significantly between ages, $F(6) = 0.663$. All values were very close to unity.

3.6.11 The effect of L-glutamate on K_d values for [^{3}H]dizocilpine binding

L-glutamate (10μM) increased the affinity of the receptor for dizocilpine from PND5-8 compared to control conditions (Table 9B). As under control conditions (Table 9A) the K_d value for [^{3}H]dizocilpine binding did not change significantly from

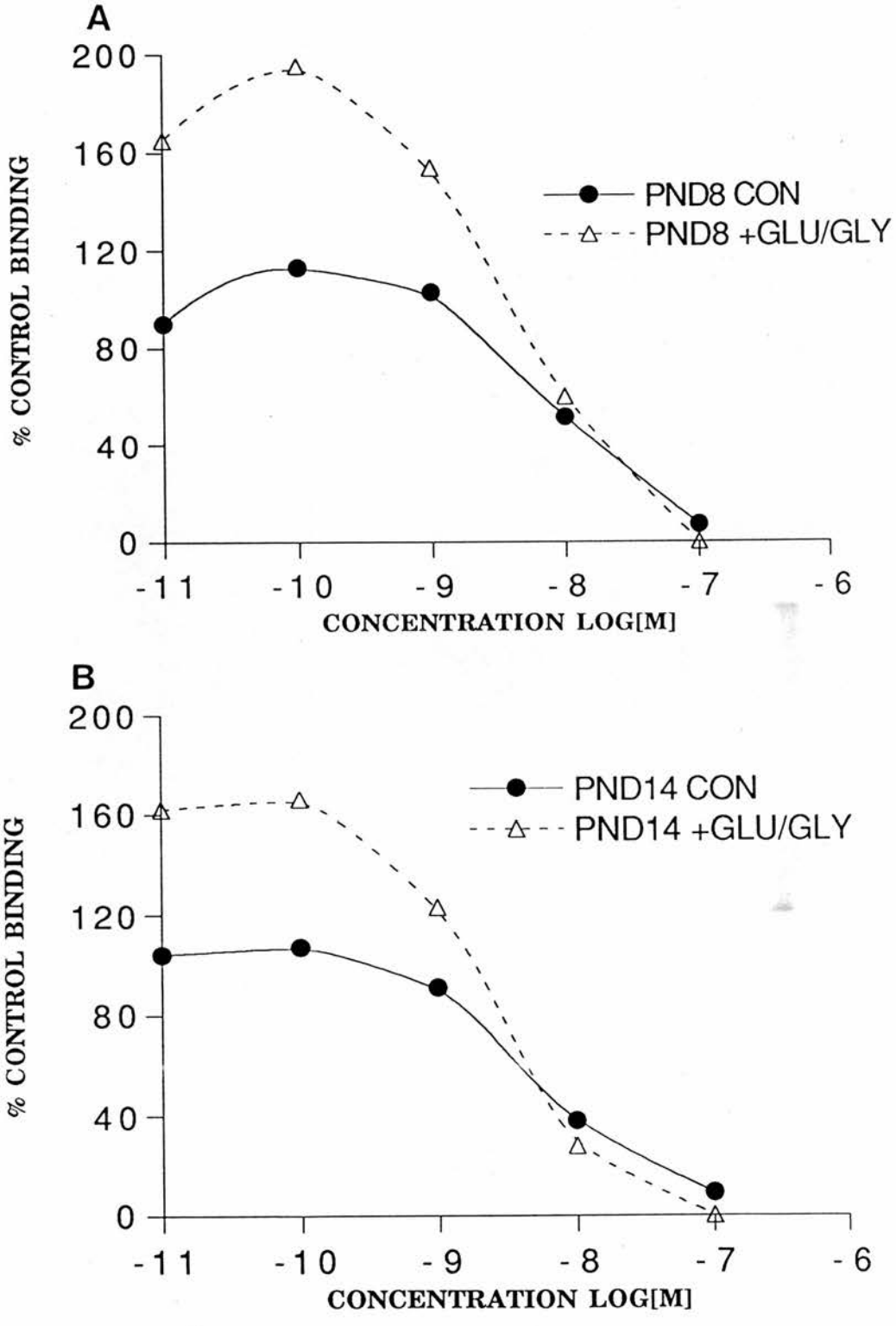


FIGURE 38: Inhibition of [^{3}H]dizocilpine binding to whole membranes at two postnatal ages

[^{3}H]dizocilpine binding was measured as previously described(Fig.31), in the presence of increasing concentrations of dizocilpine(0.01-100nM) in the absence or presence of L-glutamate and glycine(both 10μM). Binding was expressed as a percentage of binding under control conditions.

A. Binding at PND8, K_d values of 7.9nM and 5.0nM were calculated in the absence and presence of L-glutamate and glycine respectively.

B. Binding at PND14, K_d values of 3.4nM and 2.6nM were calculated in the absence and presence of L-glutamate and glycine respectively.

TABLE 9 K_d VALUES FOR [^{3}H]DIZOCILPINE BINDING TO WHOLE MEMBRANES IN THE ABSENCE OR PRESENCE OF L-GLUTAMATE

Membranes were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the presence of increasing concentrations of dizocilpine (0.01 - 100nM), in the absence (A) or presence (B) of L-glutamate (10µM). Non-specific binding was measured with dizocilpine (30µM). Data were fitted to the logistic expression $Y = MX^p/(X^p + IC_{50})$, K_d values were calculated. No significant differences between ages were revealed by one-way analysis of variance under control conditions.

A: Control

AGE RANGE (days)	K_d (nM)	n	n_H	MEAN AGE (days)
0 - 4	NM			
5 - 8	4.7 ± 0.77	8	1.02 ± 0.08	6.3
9 - 12	3.5 ± 0.56	3	1.08 ± 0.13	9.5
13 - 16	4.6 ± 0.95	6	0.99 ± 0.08	14.3
17 - 20	3.9 ± 1.56	3	0.92 ± 0.1	17.7
21 - 24	6.9 ± 1.95	3	1.37 ± 0.17	22.3
25 - 28	4.6 ± 0.54	3	1.26 ± 0.32	26.0
90	4.9 ± 0.42	15	1.11 ± 0.56	90.0

B: +L-Glutamate (10µM)

AGE RANGE (days)	K_d (nM)	n	n_H	MEAN AGE (days)
0 - 4	7.9 (7.9; 7.9)*	2	NM	1.0
5 - 8	3.6 ± 0.89	6	1.07 ± 0.08	6.4
9 - 12	3.0 ± 0.38	4	1.24 ± 0.10	9.5
13 - 16	2.8 ± 0.23	6	1.10 ± 0.03	14.3
17 - 20	2.9 ± 0.43	3	1.01 ± 0.04	17.7
21 - 24	2.8 ± 0.27	3	1.07 ± 0.08	22.3
25 - 28	2.6 ± 0.14	3	1.07 ± 0.07	26.0
90	3.5 ± 0.39	13	1.13 ± 0.08	90.0

* $P < 0.05$ when compared to PND90
Values represent mean ± s.e.m.
NM: not measured

PND5-PND90. Analysis of variance however revealed a significant difference between ages, $F(7) = 6.96$; $p > 0.05$. The K_d at PND0-4 (7.9nM; n = 2) was significantly higher than at PND90 (3.5 ± 0.4nM; n = 13) and all other ages. This is however in agreement with the lack of effect of L-glutamate modulation of specific binding at PND0-4 (Fig.33A). However specific binding was low and this may not be reliable. As under control conditions (Table 9A) n_H values were not significantly different from PND90 values during postnatal development, $F(6) = 2.103$. All n_H values were close to unity.

3.6.12 The effect of glycine on K_d values for [^{3}H] dizocilpine binding

Glycine (10μM) also increased the affinity of dizocilpine for its binding site (Table 10A), compared to control conditions throughout postnatal development. Reliable K_d values could not be measured at PND0-4. Analysis of variance revealed no significant differences between ages, $F(6) = 2.01$. The Hill Coefficients (n_H) throughout postnatal development for binding in the presence of glycine do not vary significantly, $F(6) = 1.371$. All values are close to unity.

3.6.13 The effect of L-glutamate and glycine on K_d values for [^{3}H]dizocilpine binding

L-Glutamate and glycine increased the affinity of dizocilpine for its binding site compared to control conditions (Table 10B). The K_d values calculated throughout postnatal development from PND5-PND28 do not vary from the PND90 value (2.7 ± 0.14nM; n = 13). Analysis of variance however revealed a significant difference between ages, $F(7) = 5.429$, $p < 0.01$ with the K_d at PND0-4 (4.9nM; n = 2) being significantly higher than at PND90 ($p < 0.05$). This difference corresponds to that seen in the presence of L-glutamate alone. In the presence of both amino acids n_H was close to unity at all ages. No significant differences were found between ages, $F(7) = 1.641$.

TABLE 10 K_d VALUES FOR [^{3}H]DIZOCILPINE BINDING TO WHOLE MEMBRANES IN THE PRESENCE OF GLYCINE OR GLYCINE AND L-GLUTAMATE

Membranes were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the presence of increasing concentrations of dizocilpine (0.01 - 100nM) in the presence of (A), glycine (10μM) or (B), L-glutamate and glycine (both 10μM). Non-specific binding was measured with dizocilpine (30μM). Data were fitted to the logistic equation $Y = MX^P / (X^P + IC_{50})$ from which K_d values were calculated. No significant differences were revealed with analysis of variance for binding in the presence of glycine.

A: + Glycine (10μM)

AGE RANGE (days)	K_d (nM)	n	n_H	MEAN AGE (days)
0 - 4	NM			
5 - 8	3.9 ± 0.65	6	1.07 ± 0.11	6.0
9 - 12	4.1 ± 0.68	4	1.22 ± 0.15	9.5
13 - 16	2.9 ± 0.26	6	1.05 ± 0.07	14.3
17 - 20	4.3 ± 1.2	4	0.94 ± 0.08	17.7
21 - 24	3.3 ± 0.33	3	0.96 ± 0.12	22.3
25 - 28	3.2 ± 0.3	3	1.00 ± 0.11	26.0
90	3.2 ± 0.12	13	1.14 ± 0.03	90.0

B: + L-Glutamate and Glycine (10μM)

AGE RANGE (days)	K_d (nM)	n	n_H	MEAN AGE (days)
0 - 4	4.9 (5.5; 4.3)*	2	1.15 (1.3; 1.0)	1.7
5 - 8	1.2 ± 0.54	4	1.01 ± 0.07	6.3
9 - 12	3.4 ± 0.43	4	1.34 ± 0.11	9.5
13 - 16	2.4 ± 0.11	6	1.09 ± 0.04	14.3
17 - 20	2.5 ± 0.26	4	1.07 ± 0.03	18.0
21 - 24	2.6 ± 0.04	3	1.07 ± 0.08	22.3
25 - 28	2.8 ± 0.11	3	1.09 ± 0.03	26.0
90	2.7 ± 0.14	13	1.08 ± 0.02	90.0

* $P < 0.05$ when compared to PND90
Values represent mean ± s.e.m.
NM: not measured

3.6.14 Comparison of K_d and n_H values between control and modulatory conditions

All K_d values obtained under each experimental condition were compared with each other at the same age ranges using two-way analysis of variance. This revealed that the K_d values obtained in the presence of one or both amino acids were significantly smaller than those obtained under control conditions, $F(3) = 11.497$, $p < 0.01$, indicating an increase in affinity. A similar analysis was carried out for respective n_H values. No significant differences between conditions were found, $F(3) = 0.485$.

3.6.15 Alternative evaluations of the variance in K_d

Plots of all K_d values and specific binding/B_{max} (SB/B_{max}) were constructed for each set of data. These are shown in Figs.39 and 40. Straight lines were fitted to all plots. Each had a slope close to zero indicating a lack of variance postnatally. This is in agreement with the findings using analysis of variance. Due to the low number of measurements at PND0-4, the significant effects seen with analysis of variance could not be detected. Data between PND0 and PND28 only are shown when PND90 data were included, the slopes of all lines were still close to zero.

3.6.16 B_{max} values under control conditions

These values are displayed in Fig.41. A similar postnatal profile to that observed for specific binding under control conditions (Fig.31A) was seen. Analysis of variance revealed a significant difference in receptor density between ages, $F(6) = 5.847$; $p < 0.01$. Further analysis revealed that the B_{max} at PND25-28 (mean age 26.0d; 1.096 ± 0.16pmol/mg protein; $n = 3$) was significantly higher ($p < 0.05$) than at PND90 (0.625 ± 0.05pmol/mg protein; $n = 15$). This accounts for almost 175% of the PND90 value.

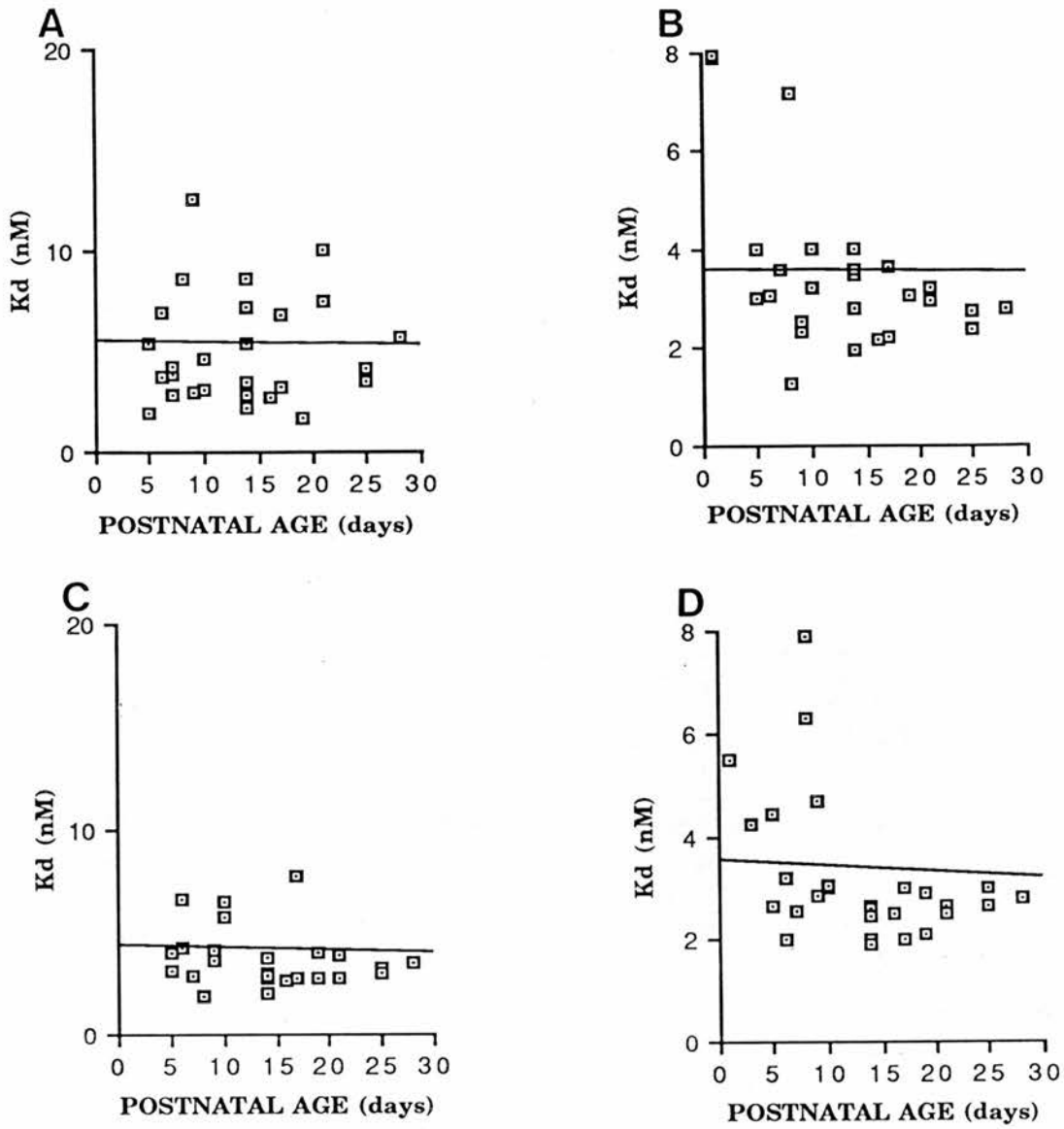


FIGURE 39: Alternative evaluation of the postnatal variance in the K_d for [^{3}H]dizocilpine binding to whole membranes. 1

Individual K_d values for [^{3}H]dizocilpine binding (PND0-28) under control(A) conditions and in the presence of L-glutamate(10μM; B), glycine(10μM; C) and both amino acids(D) have been plotted against age and fitted, using least squares, with a straight line using the equation: y=mx+c. The slope of the line may reveal an alteration in K_d with age. All lines have slopes near to zero indicating a lack of change.

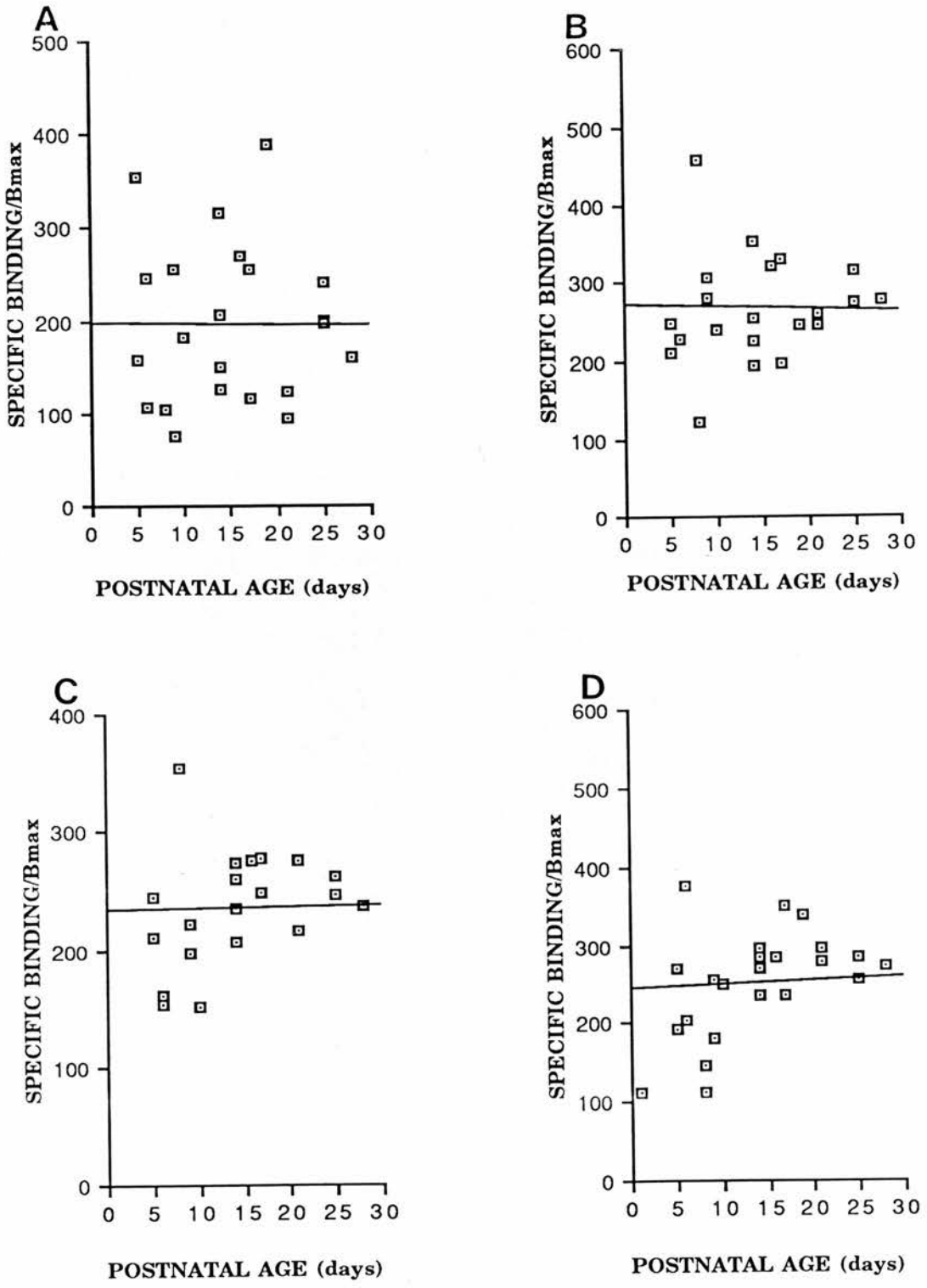
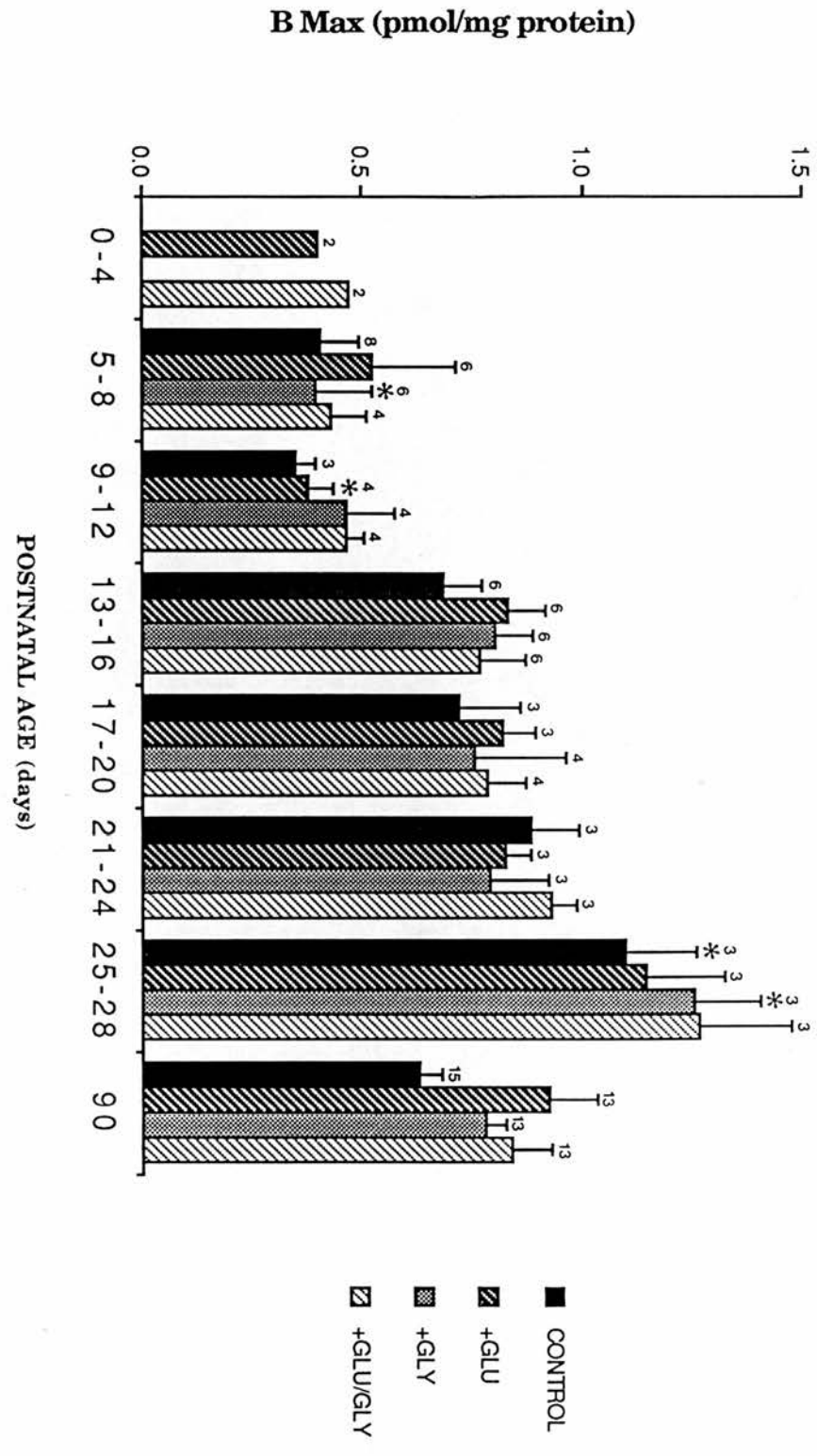


FIGURE 40: Alternative evaluation of the variance in postnatal K_d variance for [^{3}H]dizocilpine binding to whole membranes. 2

Plots of specific binding/Bmax (both calculated per mg protein) were constructed for individual data points at each postnatal age. Straight lines were fitted using the method of least squares and the equation y=mx+c. Under each condition the lines had gradients very close to zero. This may be an indication that the K_d is not altering postnatally.

FIGURE 41: Bmax values for postnatal [^{3}H]dizocilpine binding to whole membranes
Bmax values were calculated as explained in the text from specific binding(SB), ligand concentration[L] and K_d. Values were fitted to the equation: Bmax = SB (K_d + [L])/[L]. Bmax values were estimated under control conditions (CONTROL) in the presence of L-glutamate (+GLU), glycine (+GLY) and both amino acids (+GLU/GLY). Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. A significant effect was seen when $p < 0.05$, see text for full details.
*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.


B Max (pmol/mg protein)
0.0
0.5
1.0
1.5
0-4
5-8
9-12
13-16
17-20
21-24
25-28
90
POSTNATAL AGE (days)
CONTROL
+GLU
+GLY
+GLU/GLY

3.6.17 The effect of L-glutamate (10μM) on B_{max}

B_{max} values in the presence of L-glutamate (10μM) are presented in Fig.41, revealing a similar developmental profile to specific binding (Fig.32A). Lowest receptor density was measured at PND9-12 (mean age 9.5d; 0.375pmol/mg protein; n = 4) rising thereafter to a peak level at PND25-28 (mean age 26.0d; 1.14 ± 0.18pmol/mg protein; n = 3) before declining to the PND90 level (0.918 ± 0.11pmol/mg protein; n = 15). Analysis of variance revealed a significant difference between ages, $F(7) = 2.248$, $p < 0.05$. The B_{max} values at PND0-4 and PND9-12 were significantly lower than at PND90.

3.6.18 The effect of glycine (10μM) on B_{max} values

B_{max} values in the presence of glycine (10μM) are shown in Figure 41. The lowest B_{max} was at PND5-8 (mean age 6.0d; 0.391 ± 0.13pmol/mg protein; n = 6). The B_{max} thereafter increases with increasing age, as for specific binding (Fig.34A) to a maximum level at PND25-28 (mean age 26.0d; 1.25 ± 0.15pmol/mg protein; n = 3) before reaching the PND90 level (0.774 ± 0.05pmol/mg protein; n = 13). Analysis of variance revealed a significant difference between ages, $F(6) = 5.899$; $p > 0.01$, with receptor density at PND5-8 and PND25-28 being significantly different lower and higher respectively, from that at PND90 ($p > 0.05$).

3.6.19 B_{max} values in the presence of L-glutamate and glycine (both 10μM)

B_{max} values in the presence of both amino acids (Fig.41) have a similar developmental profile to that seen for specific binding (Fig.36A). Analysis of variance revealed a significant difference between ages, $F(7) = 2.916$; $p < 0.05$. No values were significantly different from the B_{max} at PND90. However, receptor density at PND0 to PND16 was significantly less than that measured at PND25-28 ($p > 0.05$).

3.6.20 Comparison of B_{max} values between control and modulatory conditions

All B_{max} values obtained under each experimental condition were compared with each other at the same age ranges using two-way analysis of variance. This revealed that the B_{max} values did not alter significantly between control and modulated conditions, $F(3) = 0.799$.

3.6.21 EC_{50} values for L-glutamate and glycine modulation of [^{3}H]dizocilpine binding

EC_{50} values for both L-glutamate and glycine were measured throughout postnatal development (Tables 11A and B). L-Glutamate modulated binding in a dose-dependent manner at all ages tested from PND5 (Fig.42A). As L-glutamate (10μM) induced increase in specific binding at PND0-4 could not be measured (Fig.33A) it was not surprising that a reproducible dose-dependent modulation in membranes prepared from this age range could not be obtained. Analysis of variance revealed a very low F value ($F(6) = 1.022$) for the EC_{50} value of L-glutamate modulation indicating no significant differences with age. These data indicate that the ability of L-glutamate to modulate [^{3}H]dizocilpine binding does not alter during postnatal development from PND5.

As for L-glutamate, glycine induced a dose-dependent modulation of [^{3}H]dizocilpine binding during postnatal development (Fig.42B) with maximum binding seen at 10μM both in immature and adult (PND90) membranes. EC_{50} values for glycine modulation during postnatal development do not vary significantly from each other as shown using analysis of variance, $F(6) = 0.407$.

3.6.22 Alternative evaluation of the variance in EC_{50} values

Plots of all EC_{50} values were made for L-glutamate and glycine with respect to age (Fig.43A and B). The lines fitted to the data have gradients very close to zero. This would indicate that the ability of L-glutamate and glycine to modulate [^{3}H]dizocilpine binding is unaltered during postnatal development. Data is shown for PND0-28 only. Inclusion of PND90 data did not influence the gradient of the line.

TABLE 11 EC_{50} VALUES FOR L-GLUTAMATE AND GLYCINE MODULATION OF [^{3}H]DIZOCILPINE BINDING TO WHOLE MEMBRANES

Membranes were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the presence of increasing concentrations of L-glutamate (A), or glycine (B), (0.01 - 100μM). Non-specific binding was measured with dizocilpine (30μM). Data were fitted to the logistic equation $Y = MX^p/(X^p + EC_{50})$. Analysis of variance revealed no significant differences between ages.

A. L-Glutamate

AGE RANGE (days)	EC_{50} (μM)	n	MEAN AGE (days)
0 - 4	NM		
5 - 8	0.18 ± 0.05	4	6.8
9 - 12	0.27 ± 0.17	4	9.5
13 - 16	0.13 ± 0.02	6	14.3
17 - 20	0.30 ± 0.09	4	18.0
21 - 24	0.15 ± 0.02	3	22.3
25 - 28	0.12(0.12; 0.12)	2	25.0
90	0.24 ± 0.03	13	90

B. Glycine

AGE RANGE (days)	EC_{50} (μM)	n	MEAN AGE (days)
0 - 4	NM	-	-
5 - 8	0.21 ± 0.07	4	7.3
9 - 12	0.13	1	9.0
13 - 16	0.10 ± 0.05	6	14.3
17 - 20	0.32 ± 0.1	3	18.3
21 - 24	0.28 ± 0.23	3	22.3
25 - 28	0.33 ± 0.21	3	26.0
90	0.23 ± 0.04	13	90

Values represent mean ± s.e.m.
NM: not measured

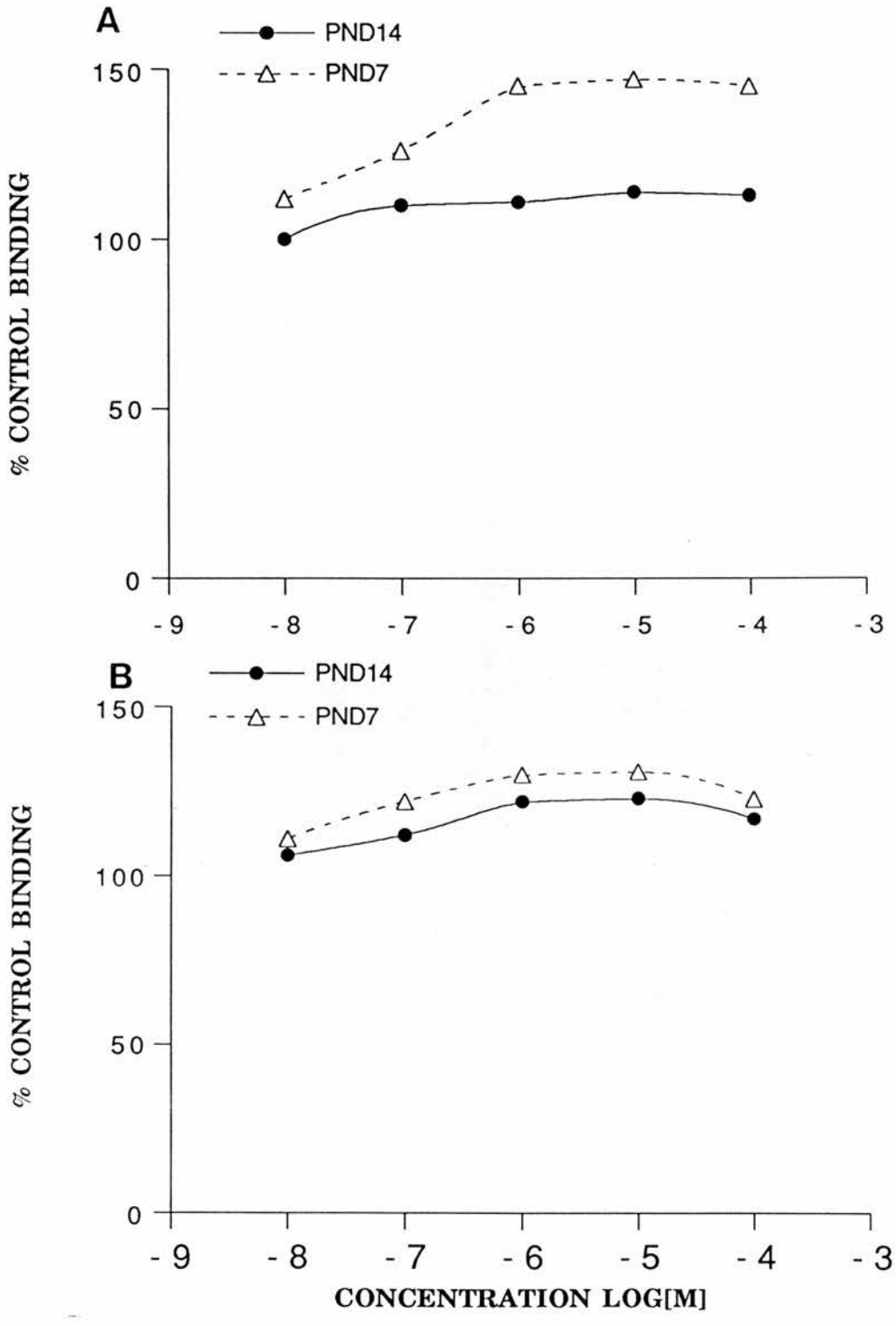


FIGURE 42: Modulation of [^{3}H]dizocilpine binding to whole membranes at two postnatal ages

Binding of [^{3}H]dizocilpine was measured as previously described in Fig.31, but in the presence of incresasing concentrations of L-glutamate(A) or glycine(B: 0.01-100μM). Binding was expressed as a percentage of binding under control conditions.

A.EC_{50} values of 0.16μM and 0.13μM were calculated for L-glutamate modulation at PND7 andPND14 respectively.

B.EC_{50} values of 0.13μM and 0.13μM were calculated for glycine modulation at PND7 andPND14 respectively.

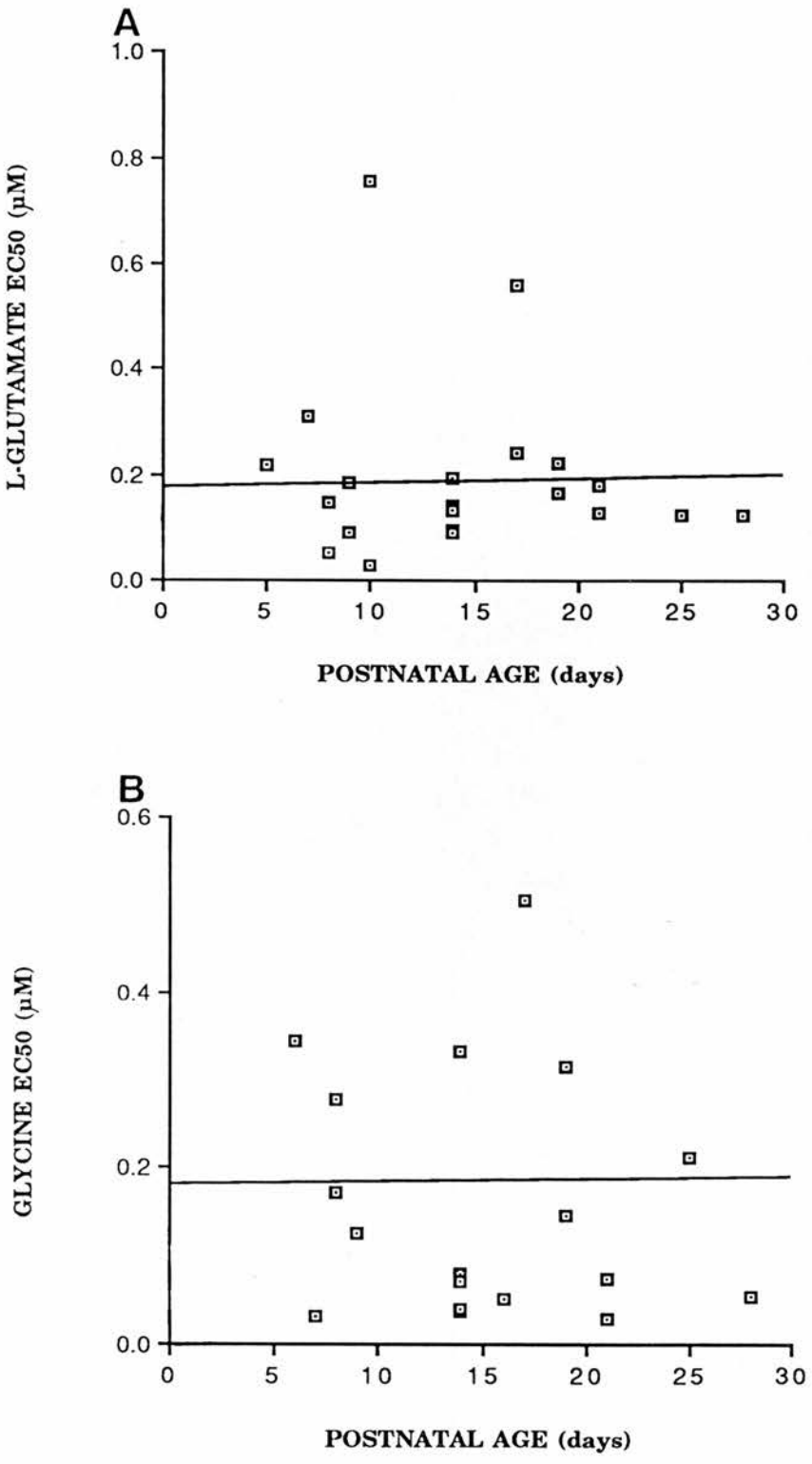


FIGURE 43: Alternative evaluation of the EC_{50} values for L-glutamate and glycine. 1
Individual EC_{50} values for L-glutamate(A) and glycine(B), have been plotted against postnatal age and fitted using the method of least squares with a straight line, using the equation; y=mx+c, to estimate the variance. Both lines have gradients close to zero indicating a lack of postnatal change.

3.7 [^{3}H]DIZOCILPINE BINDING TO SYNAPTOSOMAL MEMBRANES

3.7.1 Control conditions

Fig.44A illustrates specific binding measured with [^{3}H]dizocilpine (1nM) in synaptosomal membranes prepared freshly, from pooled cortices and hippocampi, in the absence of added L-glutamate and glycine. Binding of [^{3}H]dizocilpine, although very low, was detected at PND0-4 and at all ages tested throughout postnatal development. Specific binding gradually increased from PND0-4 (Fig.44A; mean age 0.4d; 9.35 ± 1.6 fmol/mg protein; n = 5) with increasing age until PND13-16 when a peak level of binding was reached. Analysis of variance was applied to this data, revealing a significant difference between ages, $F(6) = 6.794$; $p < 0.01$. Only binding at PND0-4 was significantly less than at PND90 ($P < 0.05$). At PND13-16 binding was significantly greater than at PND90 ($P < 0.05$).

Fig.44B illustrates specific binding but expressed per mg wet weight tissue. Binding was low at PND0-4 (mean age 0.9d; 0.095 ± 0.02 fmol/mg tissue; n = 0) increasing to peak levels at PND13-16 (mean age 14.5d; 2.77 ± 0.22 fmol/mg tissue; n = 4). This was around 195% of the adult amount (PND90; 1.75 ± 0.26 fmol/mg tissue; n = 13) and was significantly higher than binding at PND9-12. After this peak level binding decreased to PND90 levels. Analysis of variance revealed a significant difference between ages $F(7) = 7.536$; $p < 0.01$. Binding was significantly lower than at PND90 between PND0 and PND8 ($P < 0.05$). Peak binding at PND13-16 was not significantly greater than at PND90.

3.7.2 The effect of L-glutamate on [^{3}H]dizocilpine binding

Figure 45A illustrates the specific binding of [^{3}H]dizocilpine when maximally modulated by L-glutamate during postnatal development. The effect of L-glutamate was not examined at PND0-4 due to very low specific binding, making measurements unreliable. When expressed per mg protein (Figure 45A) binding increased from the lowest level at PND5-8 (mean age 6.4d; 149.5 ± 18.6 fmol/mg protein; n = 4), when it is already 50% of adult value (PND90; 298.6 ± 24.7 fmol/mg protein; n = 3). Binding

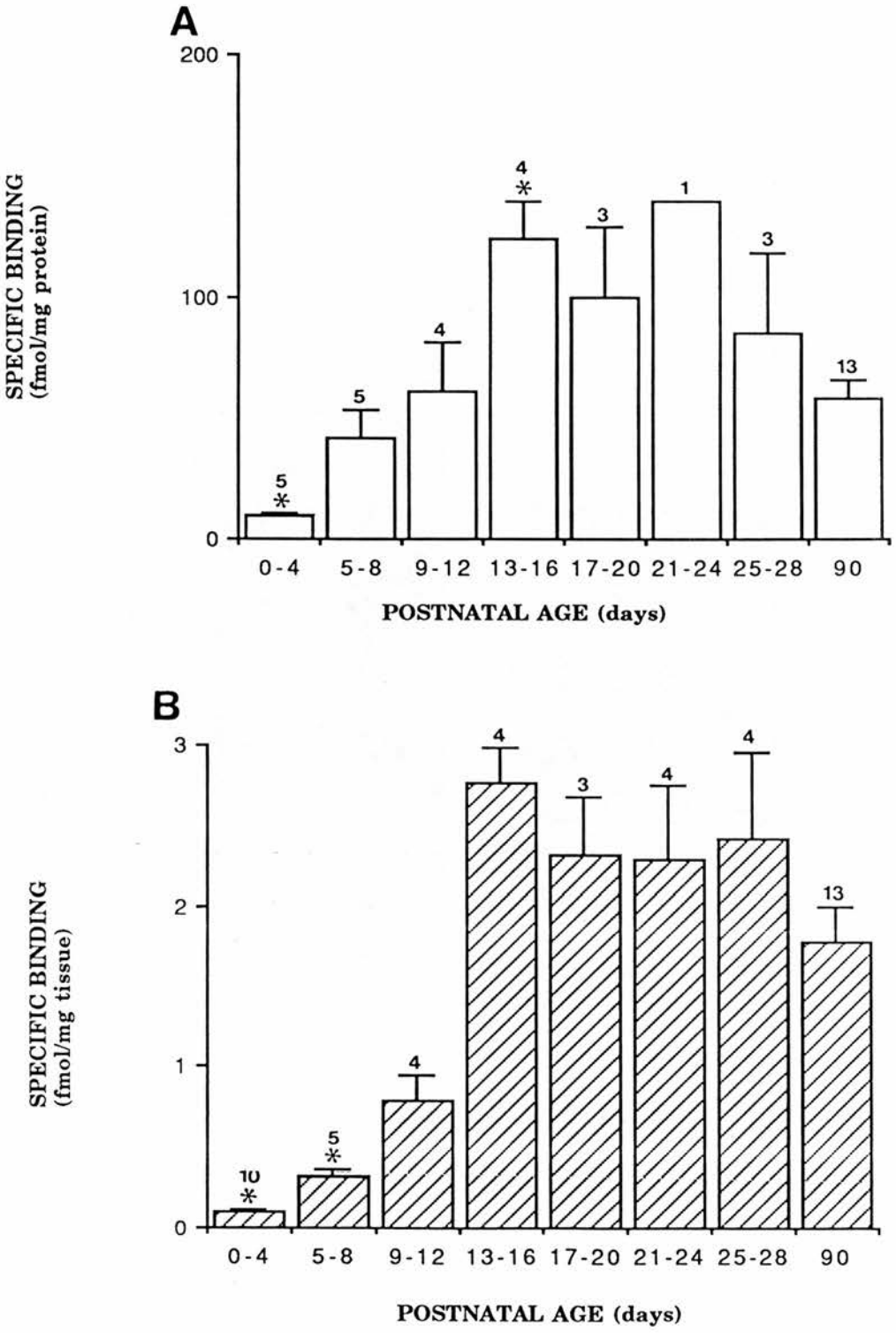


FIGURE 44: Postnatal binding of [^{3}H]dizocilpine to synaptosomal membranes
Membranes (fresh) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) in the absence or presence of dizocilpine (30μM) to measure total and non-specific binding. Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages ($p < 0.05$), see text for details. *, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

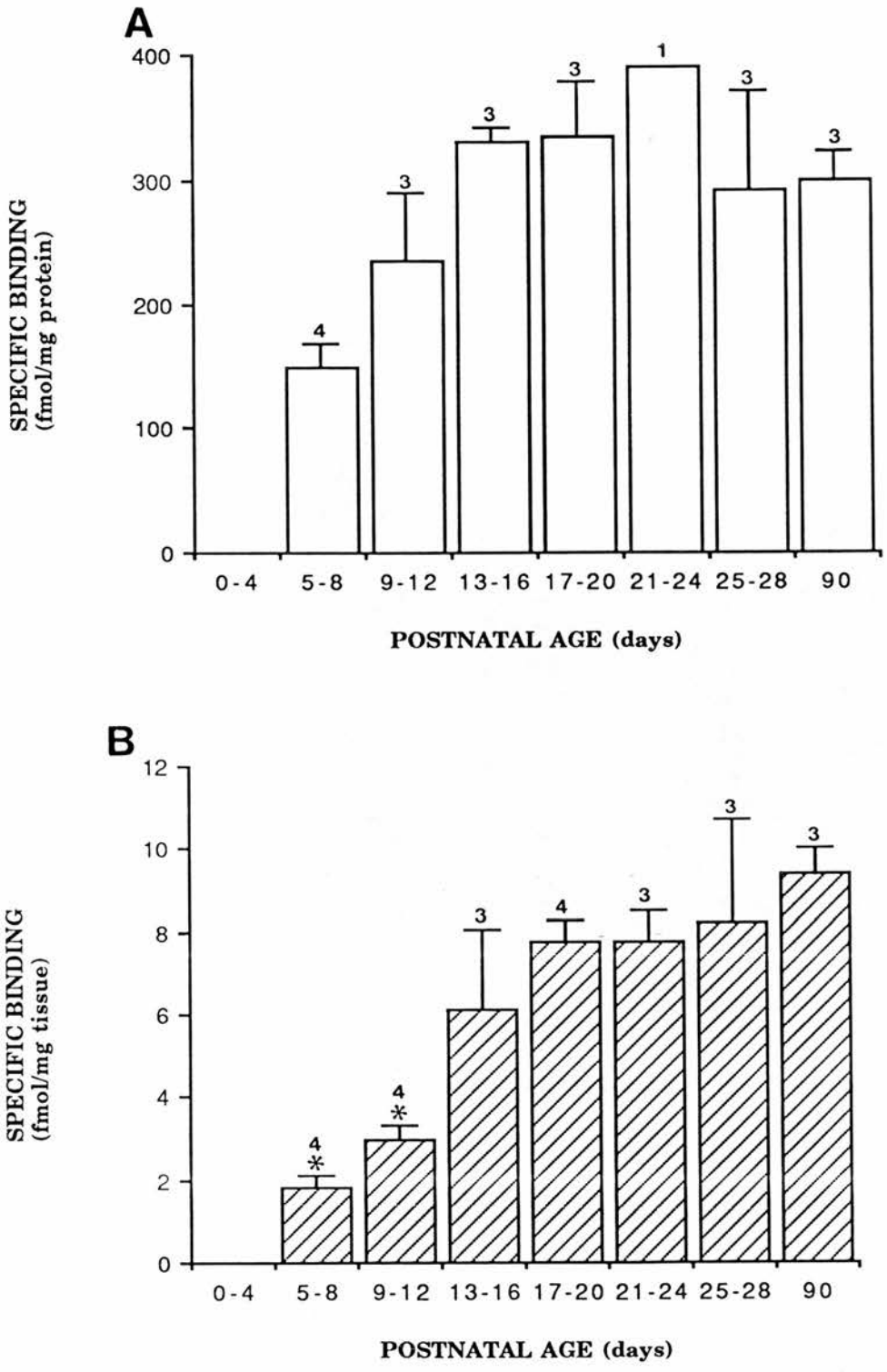


FIGURE 45: The effect of L-glutamate on the postnatal binding of [^{3}H]dizocilpine to synaptosomal membranes

Membranes (fresh) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) and L-glutamate (10μM). Dizocilpine (30μM) was used to measure non-specific binding. Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages ($p < 0.05$), see text for details. Binding was not measured at PND0-4.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

continues to increase until a peak is reached at PND21-24 (mean age 23d; 390.2 fmol/mg protein; n = 1) before adult levels are reached. This peak represents 130% of adult binding. Analysis of variance revealed no significant differences between ages, F(5) = 1.798. It must therefore be assumed that adult levels of binding are present from PND5-8 (mean age 6.4d).

When this data is expressed per mg tissue (Fig.45B) a different developmental pattern is seen. Binding of [^{3}H]dizocilpine increased gradually and with increasing age during development until PND90 levels are reached. At PND5-8 (mean age 6.4d; 1.83 ± 0.27 fmol/mg tissue; n = 4) binding is almost 20% of adult levels (PND90; 9.4 ± 0.65 fmol/mg tissue; n = 3). Analysis of variance revealed a significant difference between ages, F(6) = 5.39; $p < 0.01$. Binding at PND5-8 and 9-12 is significantly lower than PND90 binding ($p < 0.05$). At PND13-16 binding reaches a level not significantly different from that at PND90.

3.7.3 Extent of L-glutamate modulation

The maximum extent of [^{3}H]dizocilpine modulation compared to control conditions is illustrated in Fig.46. Application of analysis of variance to these data reveal no significant differences between ages. F(5) = 1.439 when expressed per mg protein and, F(6) = 1.831 when expressed per mg wet weight. L-Glutamate modulation is therefore not altering postnatally from PND5 in this membrane preparation.

3.7.4 The effect of glycine on [^{3}H]dizocilpine binding

Binding in the presence of glycine is illustrated in Fig.47. Binding was only measured from PND5-8 as it was difficult to obtain reliable results at younger ages due to the low specific binding. PND5-8 (Fig.47A; mean age 6.4d; 95.51 ± 15.9 fmol/mg protein; n = 5) binding is approximately 48% of that seen in adult membranes (PND90; 199.1 ± 15.9 fmol/mg protein; n = 3). This is almost identical to the proportion of binding measured in the presence of L-glutamate at this age range (Fig.45A). Binding increased gradually to a maximum level at PND21-24 (mean age 23d; 301.2 ± 301.2

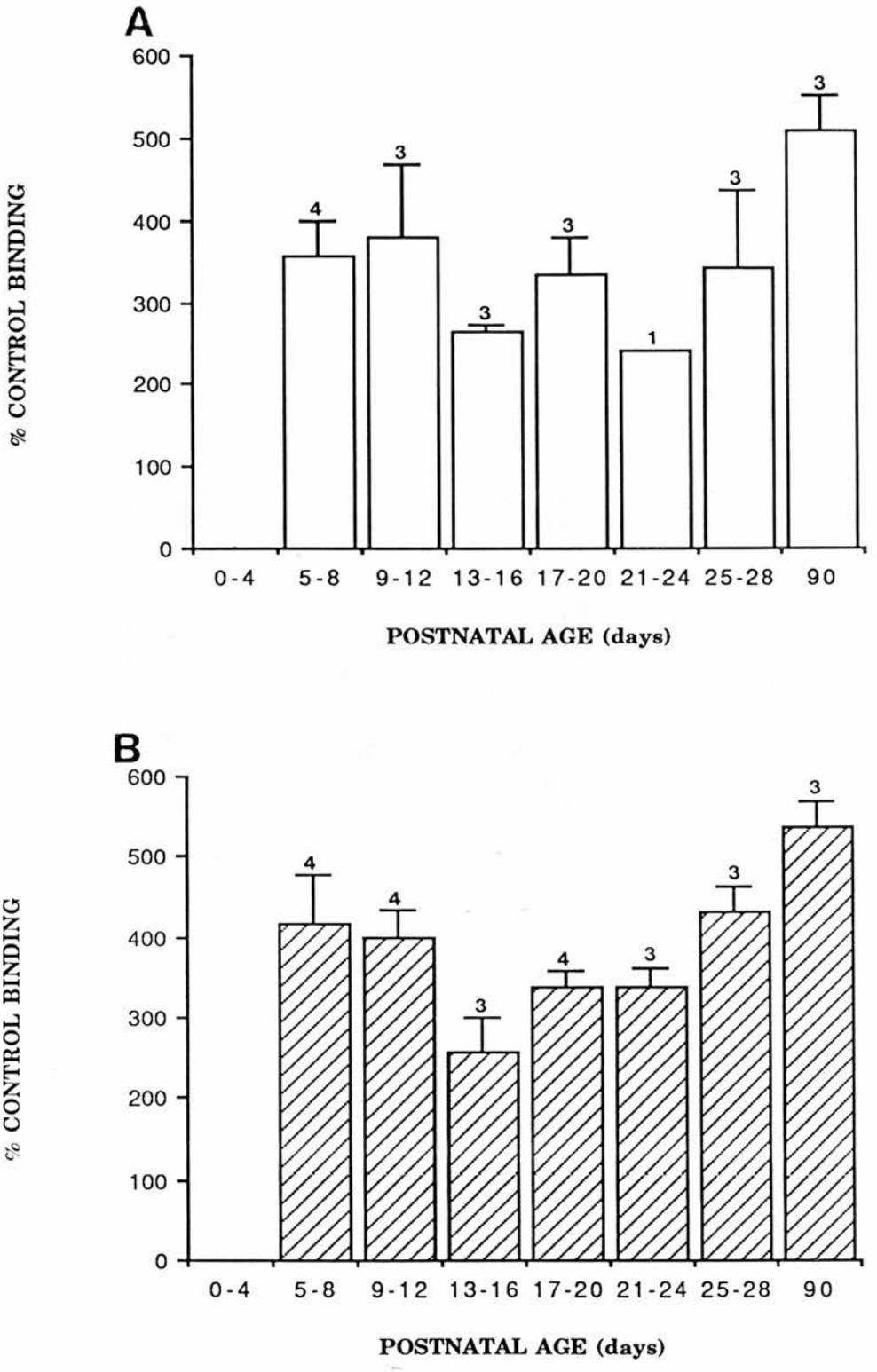


FIGURE 46: Extent of L-glutamate modulation of [^{3}H]dizocilpine binding to synaptosomal membranes

Each determination of binding in Fig.45 was expressed as a percentage of binding under control conditions both per mg protein (A) and per mg tissue (B), to evaluate whether the extent of modulation by L-glutamate altered during postnatal development. Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. No significant effects were seen between ages. L-Glutamate is therefore modulating binding to a similar extent throughout postnatal development. *, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

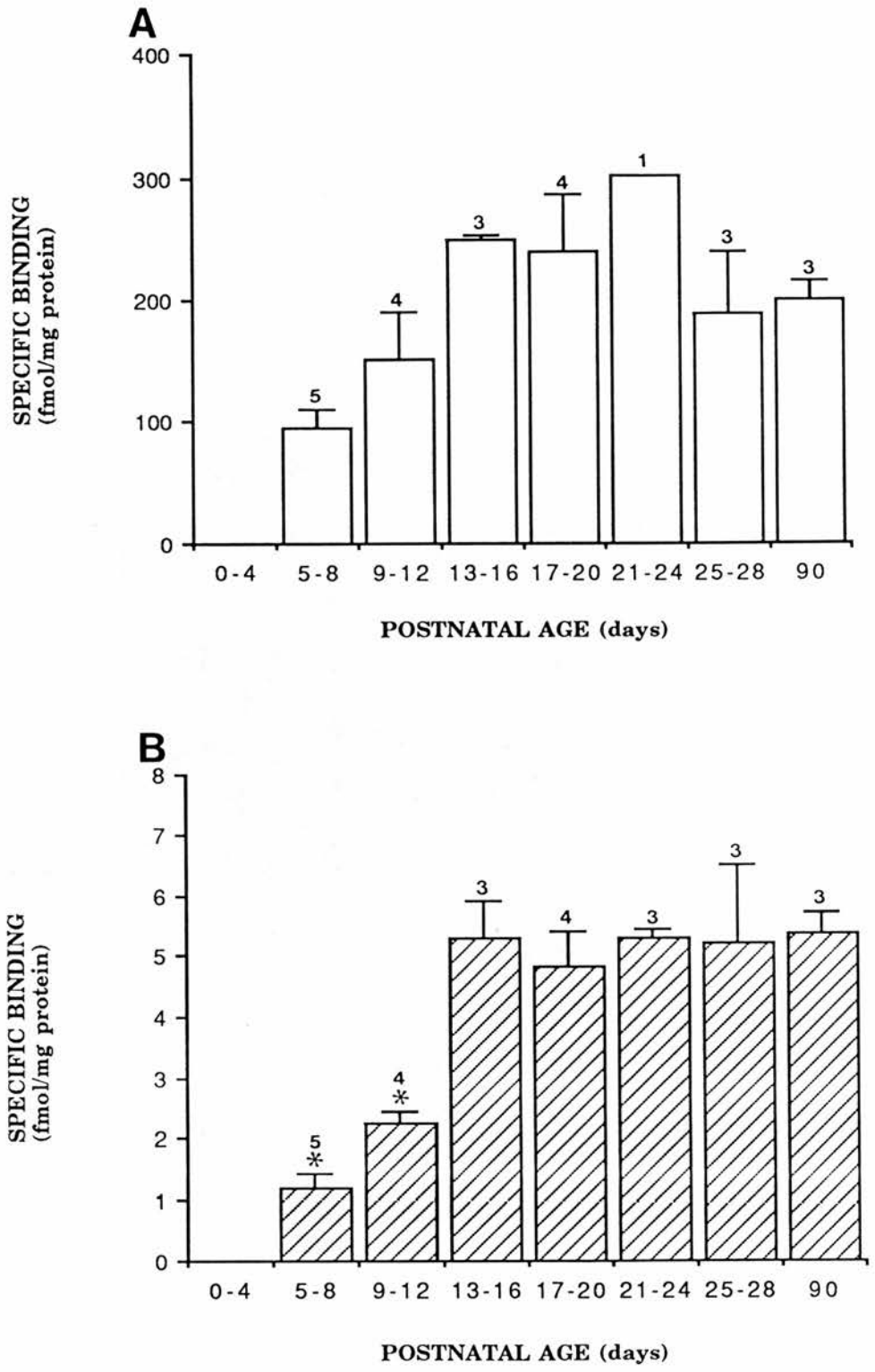


FIGURE 47: The effect of glycine on the postnatal binding of [^{3}H]dizocilpine to synaptosomal membranes

Membranes (fresh) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM) and glycine (10μM). Dizocilpine (30μM) was used to measure non-specific binding. Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages ($p < 0.05$), see text for details. Binding was not measured at PND0-4.

*, $P<0.05$ when compared to PND90 value. Number of observations are indicated above each column.

fmol/mg protein; n = 1) before falling to the adult value. Analysis of variance revealed no significant differences between ages, F(6) = 2.225, a similar profile to that seen for L-glutamate modulation. Therefore binding in the presence of glycine (10μM) as in the presence of L-glutamate (10μM) reached levels not significantly different from those at PND90 by PND5-8 (mean age 6.4d).

When expressed per mg tissue binding increased from PND5-8 to PND13-16 when a level similar to that at PND90 was reached (Fig.47B). At PND5-8 (mean age 6.4d; 1.20 ± 0.24 fmol/mg tissue) binding was approximately 20% of adult levels (5.63 ± 0.42 fmol/mg tissue; n = 3). Analysis of variance revealed a significant difference between ages, F(6) = 6.206; $p < 0.01$. Binding not significantly different from PND90 levels was reached from PND13-16 (mean age 14.5d; 5.29 ± 0.96 fmol/mg tissue; n = 3). Prior to this age, binding was significantly lower than at PND90 ($P < 0.05$).

3.7.5 Extent of glycine modulation

The extent of glycine modulation compared to control conditions is shown in Fig.48. Analysis of variance revealed no significant differences between ages, F(6) = 0.791 and F(5) = 1.068 when expressed per mg protein (Fig.48A) and per mg tissue (Fig.48B) respectively. Therefore the ability of glycine to modulate binding does not vary postnatally from PND5.

3.7.6 The effect of L-glutamate (10μM) and glycine (10μM) on [^{3}H]dizocilpine binding

Modulation of binding by both amino acids was seen throughout postnatal development. Specific binding measured in the presence of both L-glutamate and glycine is shown in Fig.49. When expressed per mg protein (Fig.49A), binding was lowest at PND0-4 (mean age 0.4d; 60.34 ± 17.4 fmol/mg protein; n = 3) representing approximately 20% of adult levels (PND90; 304.4 ± 31.8 fmol/mg protein; n = 13). There was a gradual increase in binding until maximum binding was reached at PND13-16 (mean age 14.5d; 307.8 ± 28.2 fmol/mg protein; n = 4), which was similar

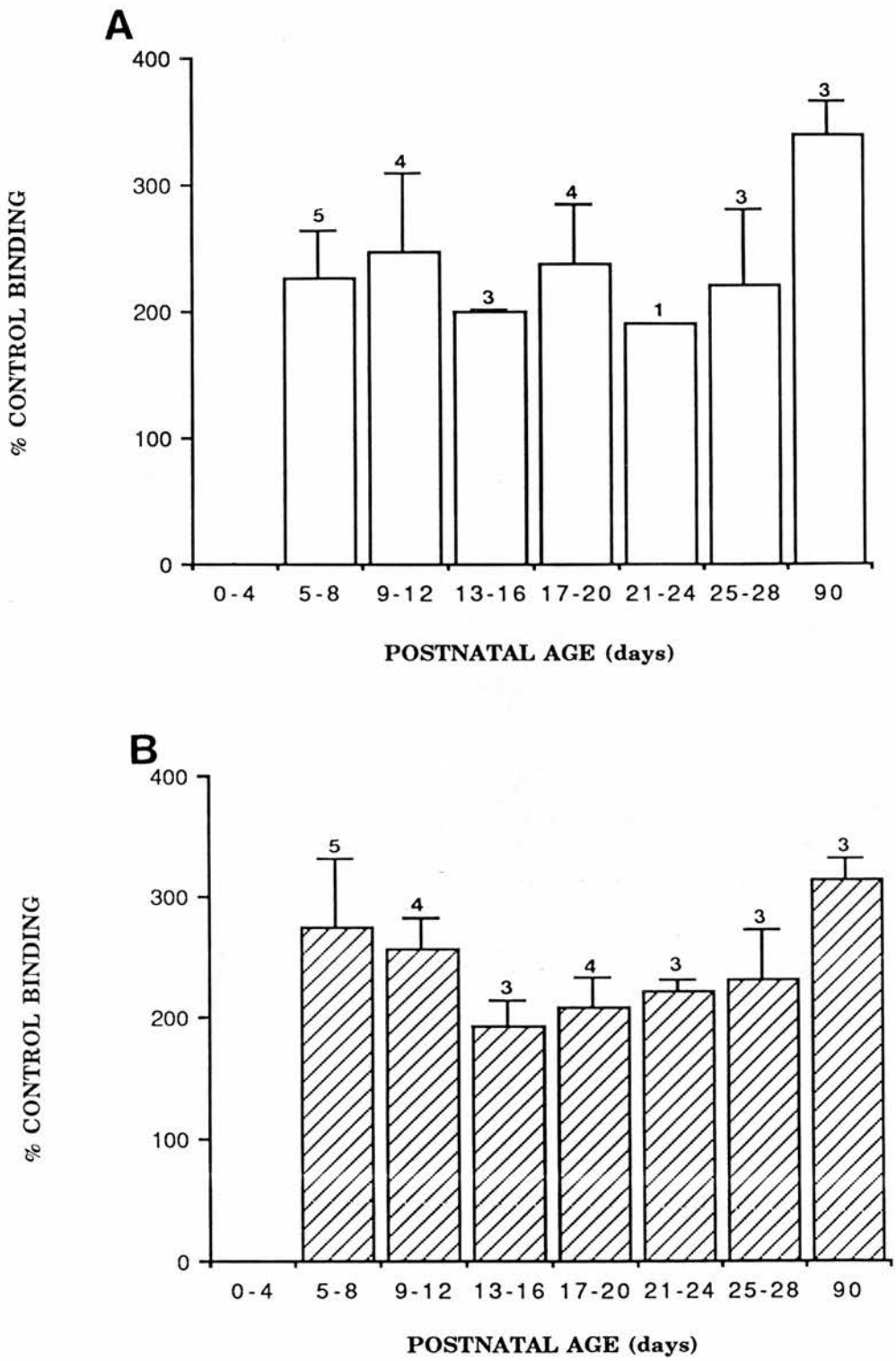


FIGURE 48: Extent of glycine modulation of [^{3}H]dizocilpine binding to synaptosomal membranes

Each determination of binding in Fig.47 was expressed as a percentage of binding under control conditions both per mg protein (A) and per mg tissue (B), to evaluate whether the extent of modulation by glycine altered during postnatal development. Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. No significant effects were seen between ages. Glycine is therefore modulating binding to a similar extent throughout postnatal development.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

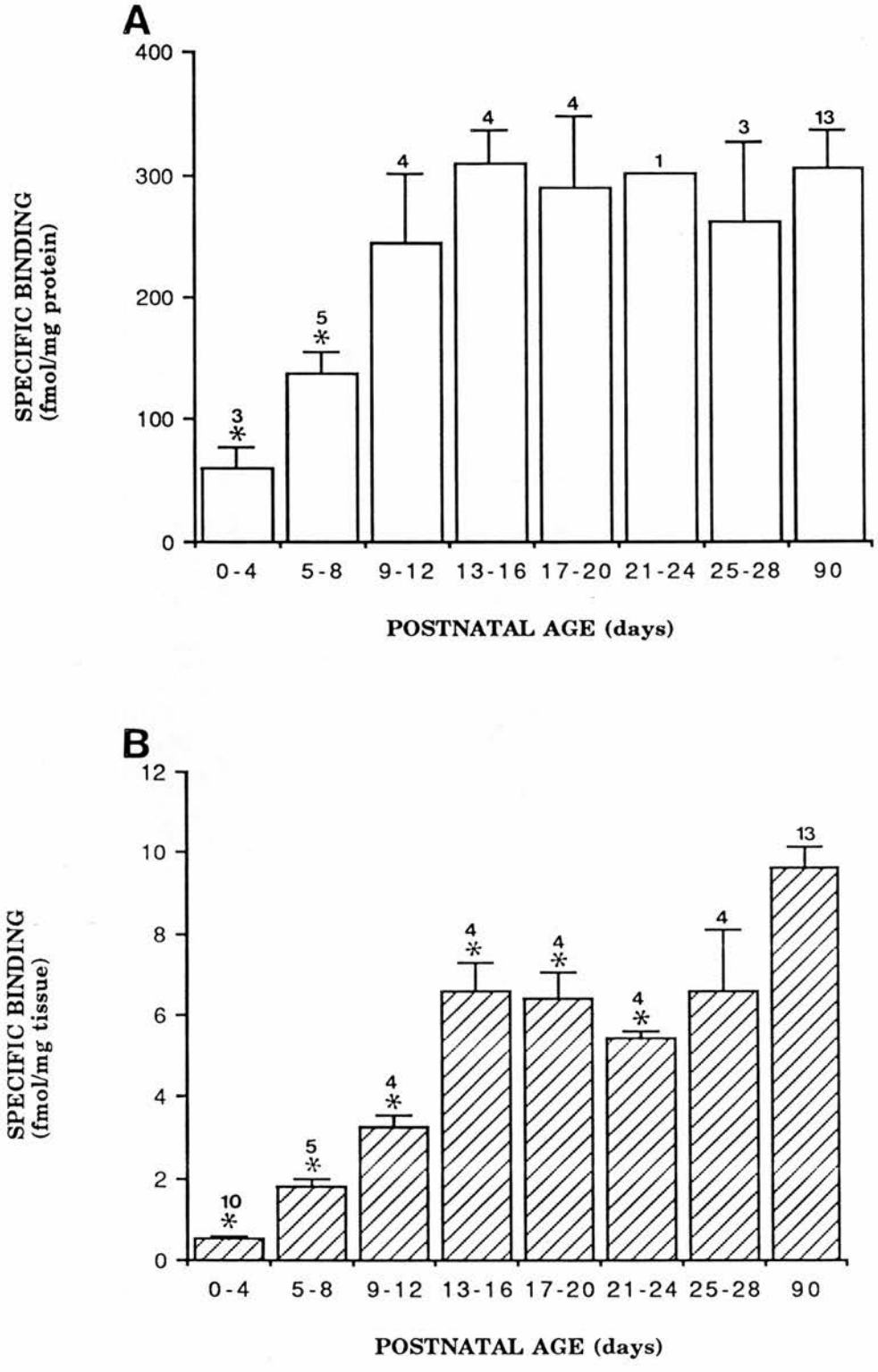


FIGURE 49: The effect of L-glutamate and glycine on the postnatal binding of [^{3}H]dizocilpine to synaptosomal membranes

Membranes (fresh) were incubated at 25°C for 45 min with [^{3}H]dizocilpine (1nM), L-glutamate and glycine (both 10μM). Dizocilpine (30μM) was used to measure non-specific binding. Specific binding was calculated and expressed per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis using one-way analysis of variance followed by a t-test revealed a significant difference in binding between different ages ($p < 0.05$),see text for details.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

to adult binding. Analysis of variance revealed a significant difference between ages, F(6) 4.518; $p < 0.01$. Binding between PND0 and PND8 was significantly lower than that at PND90 ($p < 0.05$). Adult levels were therefore reached from PND9-12.

When expressed per mg tissue (Fig.49B), binding is lowest at PND0-4 (mean age 0.9d; 0.94 ± 0.07 fmol/mg tissue; $n = 10$). Binding increased to a maximum level at PND90 (9.69 ± 0.52 fmol/mg tissue; $n = 13$). Analysis of variance revealed a significant difference between ages $F(7) = 13.795$; $p < 0.01$. Further analysis revealed that binding was significantly lower than PND90 levels between PND0 and PND24 ($p < 0.05$) apart from PND25-28.

3.7.7 Extent of modulation by L-glutamate and glycine

The extent of modulation by both amino acids is shown in Fig.50. Expressed per mg protein, analysis of variance revealed a significant difference between ages, $F(6) = 3.541$; $p < 0.05$. The extent of modulation seen at PND0-4 was significantly higher from that at all other ages except PND90 ($p < 0.05$). Binding at PND90 was significantly greater than that between PND13 and PND28 ($p < 0.05$). Expressed per mg tissue a significant difference between ages was also found; $F(7) = 4.31$; $p < 0.01$. Binding between PND13 and PND28 was significantly lower from that at PND90 ($p < 0.05$) and PND0-4 ($p < 0.05$).

3.7.8 Comparison of specific binding between control and modulatory conditions

Specific binding at each age was compared with binding in each other condition using two way analysis of variance. Data expressed per mg protein revealed a significant difference between conditions, $F(3) = 36.287$; $p < 0.01$. Further analysis revealed that specific binding in the presence of L-glutamate and/or glycine was significantly greater than that seen under control conditions ($p < 0.01$). A similar finding was seen for data expressed per mg tissue with $F(3) = 39.165$; $p < 0.01$. L-Glutamate and/or glycine are therefore significantly increasing binding compared to control conditions throughout postnatal development.

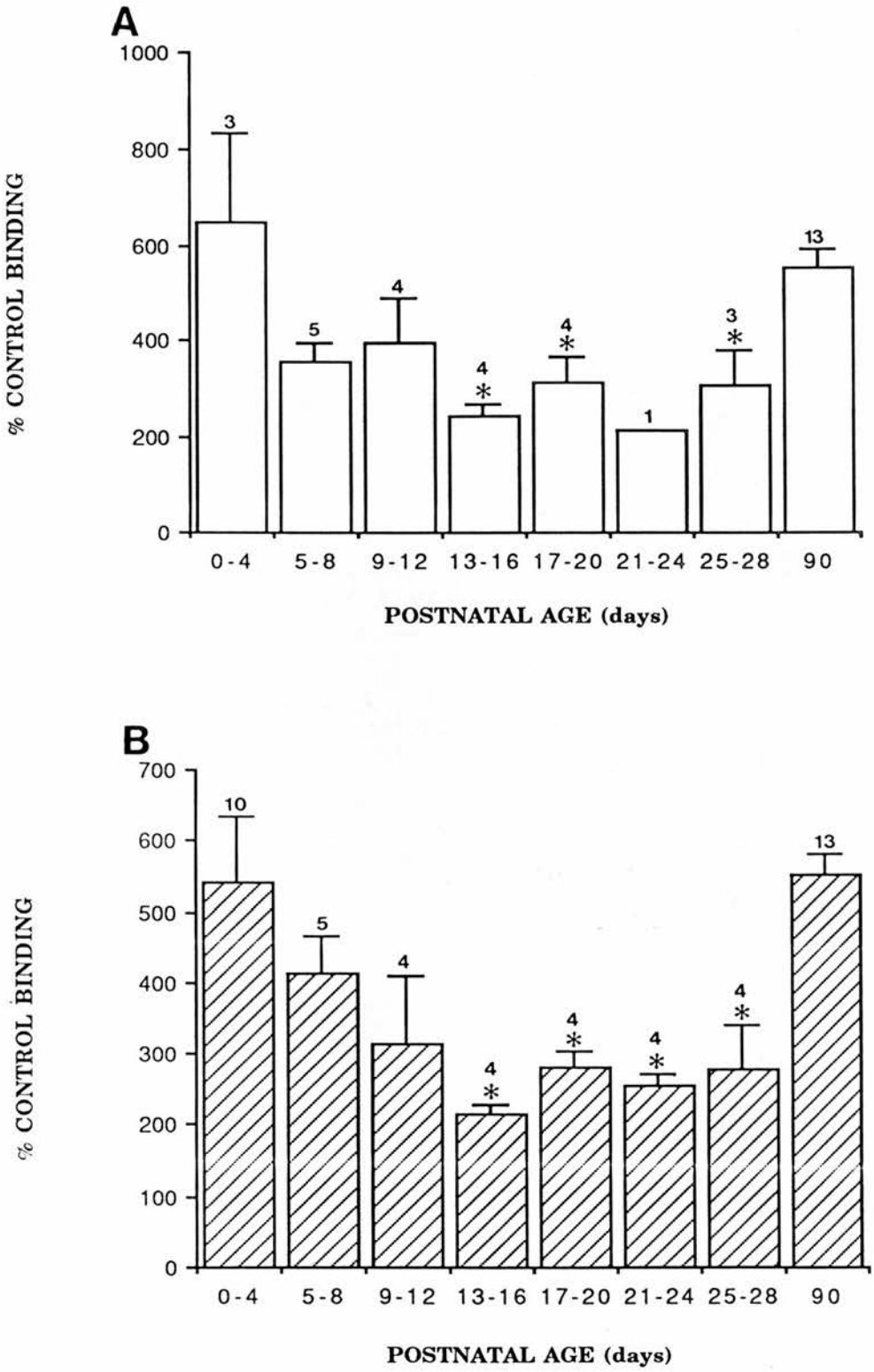


FIGURE 50: Extent of L-glutamate and glycine modulation of [^{3}H]dizocilpine binding to synaptosomal membranes

Each determination of binding in Fig.49 was expressed as a percentage of binding under control conditions both per mg protein (A) and per mg tissue (B), to evaluate whether the extent of modulation by both L-glutamate and glycine altered during postnatal development. Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. A significant effect was seen when $p < 0.05$, see text for full details.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

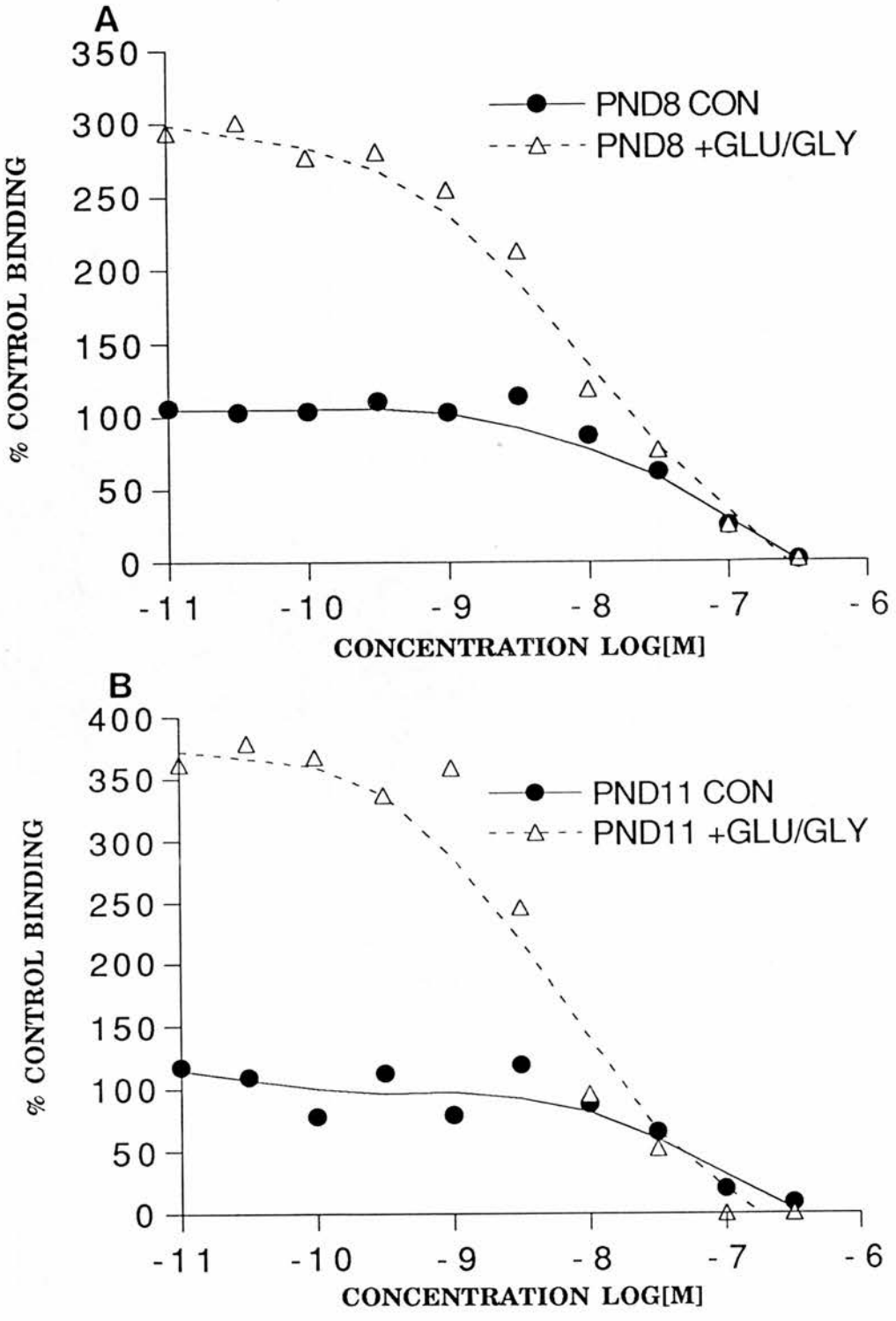


FIGURE 51 : Inhibition of [^{3}H]dizocilpine binding to syaptosomal membranes at two postnatal ages

[^{3}H]dizocilpine binding was measured as previously described(Fig.44), in the presence of increasing concentrations of dizocilpine(0.01-300nM) in the absence or presence of L-glutamate and glycine(both 10μM). Binding was expressed as a percentage of binding under control conditions.

A. Binding at PND8, K_d values of 15.5nM and 8.5nM were calculated in the absence and presence of L-glutamate and glycine respectively.

B. Binding at PND11, K_d values of 32.5nM and 5.1nM were calculated in the absence and presence of L-glutamate and glycine respectively.

TABLE 12 K_d VALUES FOR [^{3}H]DIZOCILPINE BINDING TO SYNAPTOSOMAL MEMBRANES IN THE ABSENCE OR PRESENCE OF L-GLUTAMATE

Membranes were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the presence of increasing concentrations of dizocilpine (0.01 - 100nM), in the absence (A) or presence (B) of L-glutamate (10μM). Non-specific binding was measured with dizocilpine (30μM). For data obtained under control conditions (A), IC_{50} values were estimated graphically and K_d values were calculated using Equation 2 (Section 2.7). Data in the presence of L-glutamate (B) were fitted to the logistic expression $Y = MX^p/(X^p + IC_{50})$ from which K_d values were calculated. No significant differences between ages were revealed by one-way analysis of variance.

A: Control

AGE (postnatal days)	K_d (nM)	n	MEAN AGE (days)
0 - 4	NM		
5 - 8	20.6 ± 4.2	4	5.8
9 - 12	10.9 ± 6.7	4	10.3
13 - 16	13.2 ± 3.2	4	14.5
17 - 20	11.9 ± 5.6	4	18.8
21 - 24	19.0 ± 3.7	3	23.3
25 - 28	23.6 ± 10.6	4	27.8
90	20.8 ± 3.7	13	90

b: +L-Glutamate (10μM)

AGE RANGE (days)	K_d (nM)	n	n_H	MEAN AGE (days)
0 - 4	NM			
5 - 8	3.8 ± 0.54	4	1.25 ± 0.02	6.0
9 - 12	3.1 ± 0.69	3	0.91 ± 0.16	10.0
13 - 16	4.1 ± 0.62	3	0.96 ± 0.11	14.3
17 - 20	2.9 ± 0.19	3	0.96 ± 0.14	19.0
21 - 24	4.4	1	1.11	23.0
25 - 28	4.3 ± 0.08	3	1.12 ± 0.27	27.7
90	4.9 ± 0.19	4	0.85 ± 0.07	90

Values represent mean ± s.e.m.
NM: not measured

TABLE 13 K_d VALUES FOR [^{3}H]DIZOCILPINE BINDING TO SYNAPTOSOMAL MEMBRANES IN THE PRESENCE OF GLYCINE OR L-GLUTAMATE AND GLYCINE

Membranes were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the presence of increasing concentrations of dizocilpine (0.01 - 100nM) in the presence of (A) glycine (10μM) or (B) L-glutamate and glycine (both 10μM). Non-specific binding was measured with dizocilpine (30μM). Data were fitted to the logistic equation $Y = MX^P / (X^P + IC_{50})$ K_d values were calculated. No significant differences were revealed with analysis of variance.

A: +Glycine (10μM)

AGE RANGE (days)	K_d (nM)	n	n_H	MEAN AGE (days)
0 - 4	NM			
5 - 8	9.5 ± 2.54	4	1.11 ± 0.23	6.0
9 - 12	2.7 ± 0.69	3	0.66 ± 0.07	10.0
13 - 16	5.7 ± 0.98	3	0.94 ± 0.09	14.3
17 - 20	6.7 ± 0.79	3	1.18 ± 0.16	18.8
21 - 24	6.1	1	1.7	23.0
25 - 28	5.3 ± 1.83	3	0.97 ± 0.22	27.7
90	5.6 ± 1.14	4	0.91 ± 0.12	90

B: +L-Glutamate and glycine (10μM)

AGE RANGE (days)	K_d (nM)	n	n_H	MEAN AGE (days)
0 - 4	4.1 ± 1.1	3	0.78 ± 0.16	2.7
5 - 8	4.7 ± 1.0	6	0.98 ± 0.02	6.2
9 - 12	3.2 ± 0.7	4	1.17 ± 0.1	10.3
13 - 16	6.3 ± 1.1	4	1.14 ± 0.09	14.5
17 - 20	3.9 ± 0.6	4	0.92 ± 0.1	18.8
21 - 24	5.8 (5.3; 6.3)	2	1.09	23.0
25 - 28	5.9 ± 1.2	3	0.94 ± 0.01	27.7
90	6.8 ± 1.0	13	1.1 ± 0.06	90

Values represent mean ± s.e.m.
NM: not measured

The extent of modulation was also subjected to two way analysis of variance under each method of data expression. Expressed per mg protein significant differences were seen between treatments $F(2) = 5.429$; $p < 0.01$. The extent of modulation in the presence of glycine was significantly lower from that seen with either L-glutamate or L-glutamate and glycine ($p < 0.05$). Expressed per mg tissue $F(2) = 11.996$; $p < 0.01$. Modulation by L-glutamate and by glycine was significantly less than modulation by both amino acids ($p < 0.01$).

3.7.9 Determination of K_d values for [^{3}H]dizocilpine binding

In the absence of added L-glutamate and glycine binding was very low at PND0-4 (see Fig.44), consequently K_d values for [^{3}H]dizocilpine binding were not calculated (Table 12A). At all other ages inhibition of [^{3}H]dizocilpine was measured in the absence and presence of L-glutamate and glycine (Fig.51). K_d values were calculated using Equation 2 (see Section 2.7). Application of analysis of variance to the K_d from each age range revealed no significant differences between ages, $F(6) = 0.622$.

3.7.10 The effect of L-glutamate on the K_d values for [^{3}H]dizocilpine binding

The K_d values in the presence of L-glutamate are presented in Table 12B. As for whole membranes, analysis of variance revealed no significant differences between ages, $F(5) = 2.036$. The Hill Co-efficients (n_H) at all ages are very close to unity and do not significantly differ from each other, $F(6) = 0.798$.

3.7.11 The effect of glycine on the K_d values for [^{3}H]dizocilpine binding

K_d values for [^{3}H]dizocilpine binding in the presence of glycine during postnatal development are presented in Table 13A. Analysis of variance revealed no significant differences between ages $F(5) = 1.127$. The Hill Co-efficients (n_H) were not significantly different between ages $F(5) = 1.563$.

3.7.12 The effect of L-glutamate and glycine on the K_d [^{3}H]dizocilpine binding

Inhibition of [^{3}H]dizocilpine binding was measured in the presence of both amino acids (Fig.51) K_d values were measured (Table 13B). K_d values were estimated at PND0-4 due to the higher levels of binding in the presence of both amino acids. Analysis of variance revealed that K_d did not alter significantly between ages $F(7) = 0.698$. In the presence of both amino acids n_H also does not vary significantly between ages, $F(7) = 0.985$.

3.7.13 Comparison of K_d and n_H values between control and modulatory conditions

All K_d and n_H values obtained under each experimental condition at each age range were compared with each other using two-way analysis of variance. This revealed that the K_d values obtained in the presence of L-glutamate and/or glycine were significantly smaller than in control conditions, $F(3) = 6.179$, $p < 0.01$. A similar analysis was carried out for n_H values. No significant differences between conditions were found, $F(2) = 0.140$.

3.7.14 Alternative evaluation of the variance in K_d

Plots of all K_d values and SB/B_{max} values were constructed for each set of data (Figs.52 and 53). Straight lines were fitted to all plots. For plots of all K_d values the line of best fit had a gradient close to zero in all conditions, apart from control and in the presence of L-glutamate. This is also apparent for the plots of SB/B_{max} under control conditions and in the presence of L-glutamate. It is possible therefore that the K_d is changing postnatally although analysis of variance has not detected it. Data between PND0 and PND28 only is shown. When PND90 data were included, the slopes of the lines were very similar.

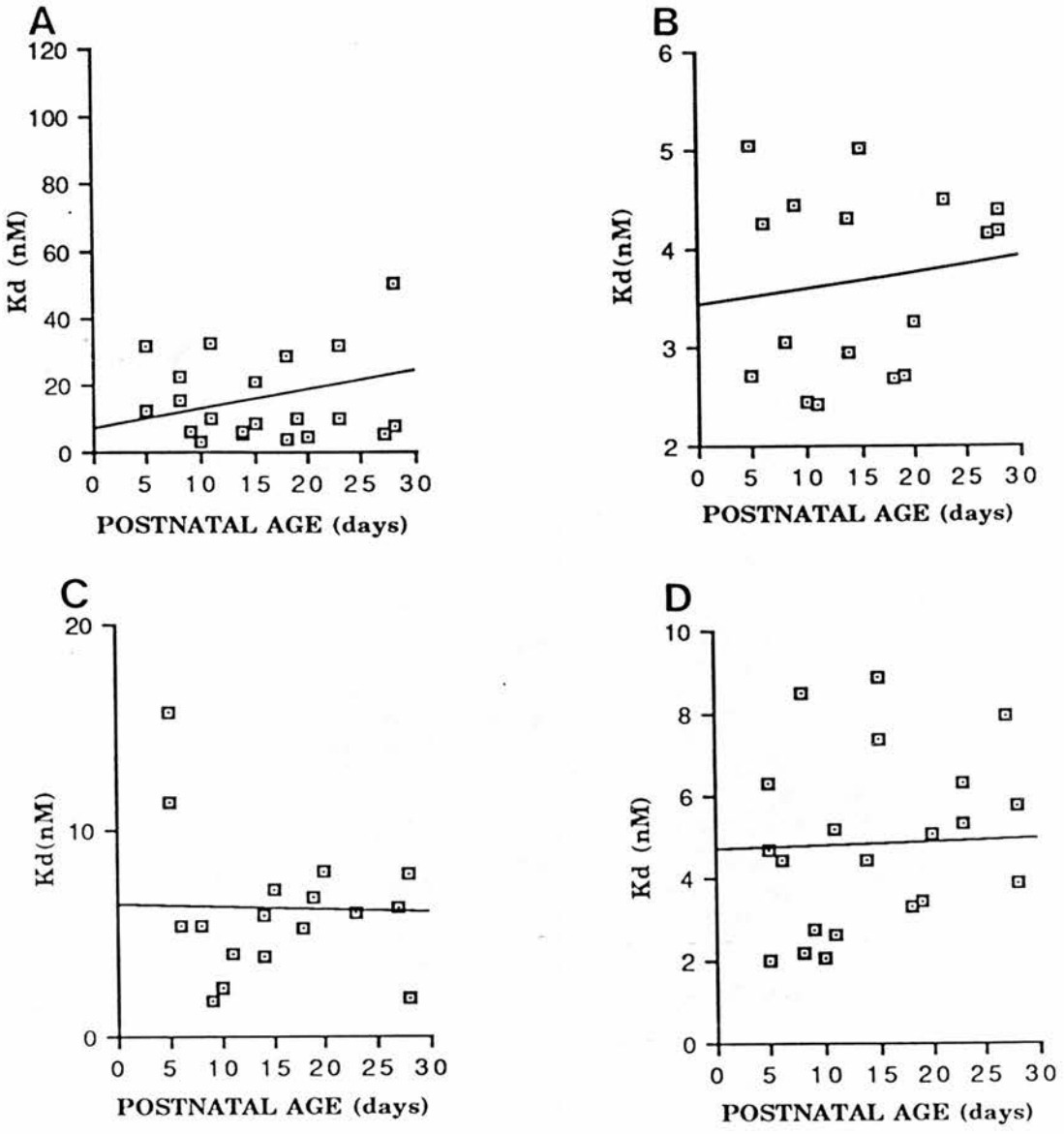


FIGURE 52 : Alternative evaluation of the postnatal variance in the K_d for [^{3}H]dizocilpine binding to synaptosomal membranes. 1

Individual K_d values for [^{3}H]dizocilpine binding (PND0-28) under control(A) conditions and in the presence of L-glutamate(10μM; B), glycine(10μM; C) and both amino acids(D) have been plotted against age and fitted, using least squares, with a straight line(using the equation: y=mx+c). The slope of the line may reveal an alteration in K_d with age. In the absence of added amino acids(A) and in the presence of L-glutamate lines with slight slopes are seen, this may indicate a postnatal change in receptor affinity. In both other cases (B) and (C) the lines of best fit have slopes close to zero indicating a lack of change.

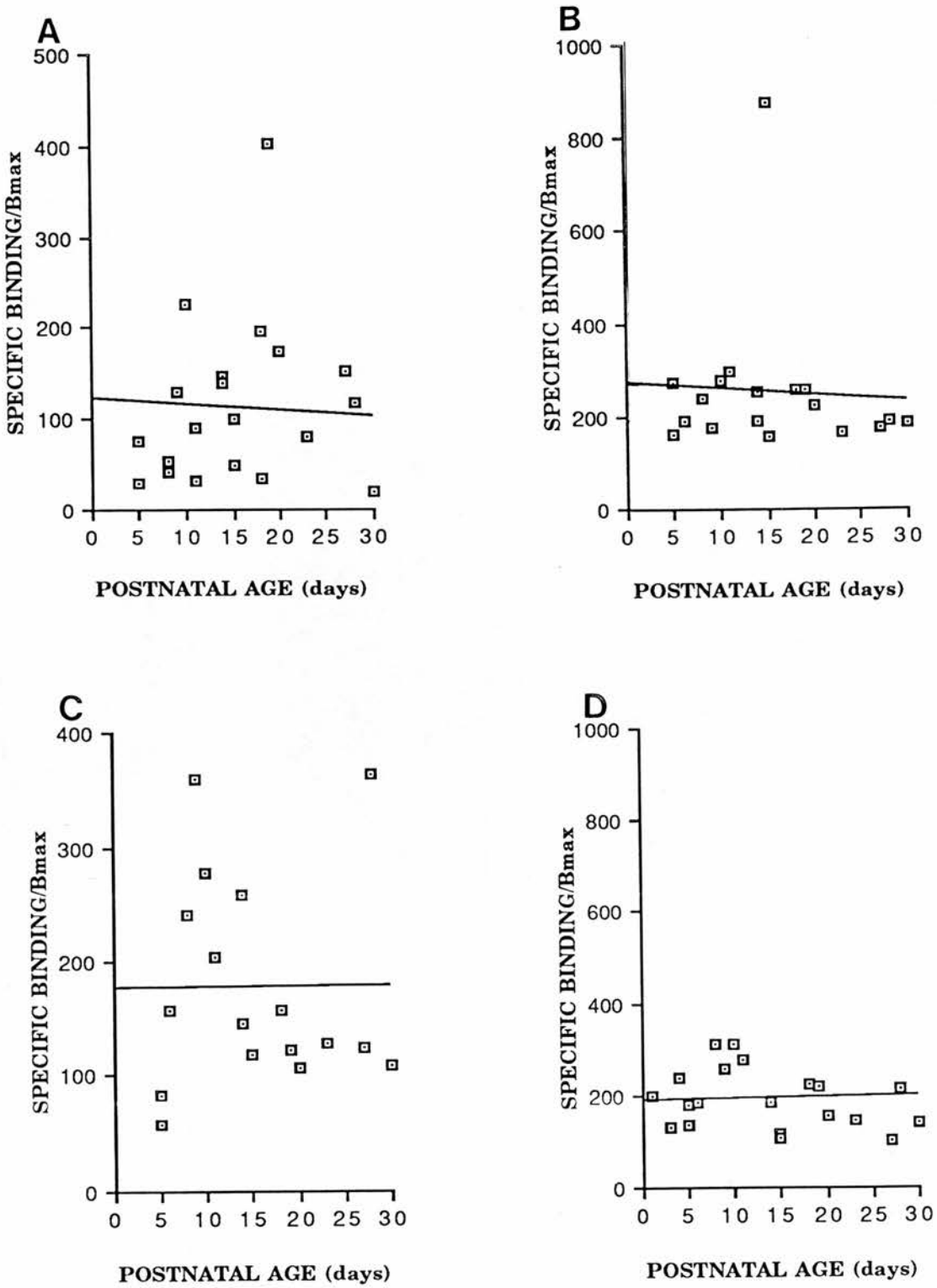


FIGURE 53: Alternative evaluation of the variance in postnatal K_d for [^{3}H]dizocilpine binding to synaptosomal membranes. 2

Plots of specific binding/Bmax (both calculated per mg protein) were constructed for individual data points at each postnatal age. Straight lines were fitted using the method of least squares to the equation: y=mx+c. In the absence (A) and presence(B) of L-glutamate lines with very slight gradients were found. This may indicate postnatal change in K_d. Both other lines (C) and (D) have gradients close to zero indicating a lack of postnatal change.

3.7.15 B_{max} values under control conditions

B_{max} values for [^{3}H]dizocilpine binding in the absence of added L-glutamate and glycine were calculated (Fig.54). Analysis of variance was applied to this data and no significant differences between ages (PND5-PND90) were found, $F(5) = 0.455$. This would suggest that adult levels of receptors are present from PND5-8 (mean age 6.0d; 0.89 ± 0.24 pmol/mg protein; $n = 4$). This is the age when corresponding specific binding reached adult levels.

3.7.16 The effect of L-glutamate on B_{max} values

Corresponding B_{max} values for [^{3}H]dizocilpine binding modulated by L-glutamate are shown in Fig.54. B_{max} values increase from 0.64 ± 0.08 pmol/mg protein ($n = 4$) at PND5-8 (mean age 6.0d) to 1.71 ± 0.29 pmol/mg protein ($n = 3$) at PND13-16 (mean age 14.3d), a value similar to that seen at PND90 (1.74 ± 0.25 pmol/mg protein; $n = 3$). Analysis of variance revealed a significant difference between ages, $F(6) = 3.393$; $p < 0.05$. B_{max} values at PND5-8 and PND9-12 were significantly lower than at PND90 ($p < 0.05$). From PND13-16 B_{max} was not significantly different from that at PND90.

3.7.17 The effect of glycine on B_{max} values

The B_{max} values for [^{3}H]dizocilpine binding in the presence of glycine are shown in Fig.54. Analysis of variance revealed a significant difference between ages $F(5) = 3.010$; $p < 0.05$. No B_{max} is significantly different from that seen at PND90 (1.18 ± 0.26 pmol/mg protein). However,B_{max} values between PND17 and PND24 are significantly higher than seen at PND5-8 and PND9-12 ($p < 0.05$).

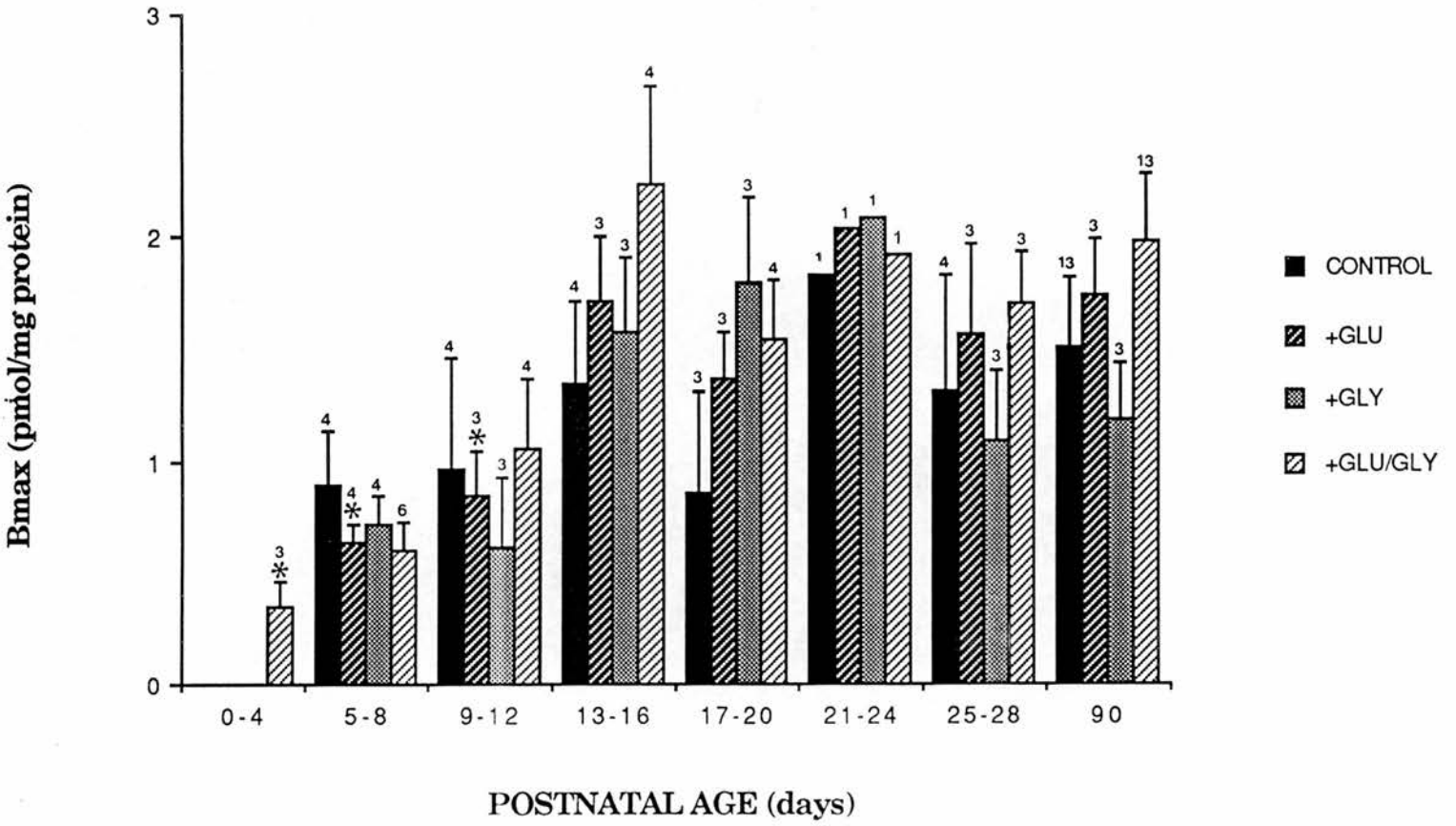
3.7.18 The effect of L-glutamate and glycine on B_{max} values

Fig.54 shows the B_{max} values for [^{3}H]dizocilpine binding modulated by both amino acids. B_{max} values are low at PND0-4 (mean age 2.7d; 0.33 ± 0.12 pmol/mg protein; $n = 3$) rising to a peak at PND13-16 (mean age 14.5d; 2.24 ± 0.44 pmol/mg

FIGURE 54: Postnatal Bmax values calculated for [^{3}H]dizocilpine binding in synaptosomal membranes

B_{max} values were calculated as explained in the text from specific binding (SB), ligand concentration[L] and K_d. Values were fitted to the equation: Bmax = SB (K_d+ [L])/[L]. Bmax values were estimated under control conditions(CONTROL), in the presence of L-glutamate (+GLU), glycine (+GLY) and both amino acids (+GLU/GLY). Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. A significant effect was seen when $p < 0.05$, see text for full details.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.


Bmax (pmol/mg protein)
3
2
1
0
0-4
5-8
9-12
13-16
17-20
21-24
25-28
90
POSTNATAL AGE (days)
CONTROL
+GLU
+GLY
+GLU/GLY

protein; n = 4), before reaching adult levels (PND90; 1.98 ± 0.3 mol/mg protein; n = 13). Analysis of variance revealed a significant difference between ages $F(6) = 3.531$; $p < 0.01$. Only the B_{max} at PND0-4 is significantly different from that at PND90 ($p < 0.05$). The B_{max} at PND13-16 is however significantly higher than those seen up to PND12 and between PND17-20 ($p < 0.05$).

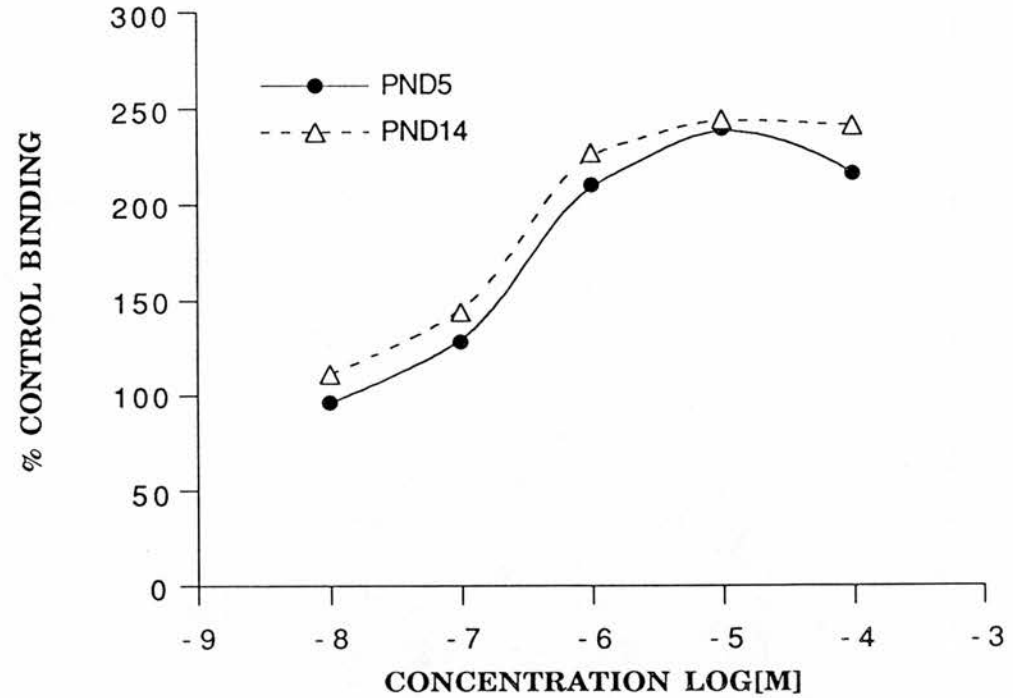
3.7.19 Comparison of B_{max} values between control and modulatory conditions

Two-way analysis of variance was used to compare B_{max} values at each age range under all conditions with each other. This revealed that $F(3) = 0.631$. Therefore B_{max} values for [^{3}H]dizocilpine binding to synaptosomal membranes are not significantly altered from control conditions by the allosteric modulators L-glutamate and glycine.

3.7.20 EC_{50} values for L-glutamate and glycine modulation of [^{3}H]dizocilpine binding to washed synaptosomal membranes

Reproducible dose-dependent modulation was not observed at PND0-4 therefore no data is available for this age range. Maximum modulation was seen in the presence of 10μM L-glutamate and glycine both in adult (Fig.17) and immature tissue (Fig.55). EC_{50} values for L-glutamate modulation were calculated (Table 14A). Analysis of variance revealed no significant differences between ages $F(6) = 0.991$.

Reproducible modulation was not easily measured at PND0-4 for glycine. EC_{50} values from PND5 onwards are displayed in Table 14B. Analysis of variance revealed no significant differences between ages during postnatal development, $F(6) = 2.168$. This is an indication that the ability of L-glutamate and glycine to modulate [^{3}H]dizocilpine binding does not alter postnatally.



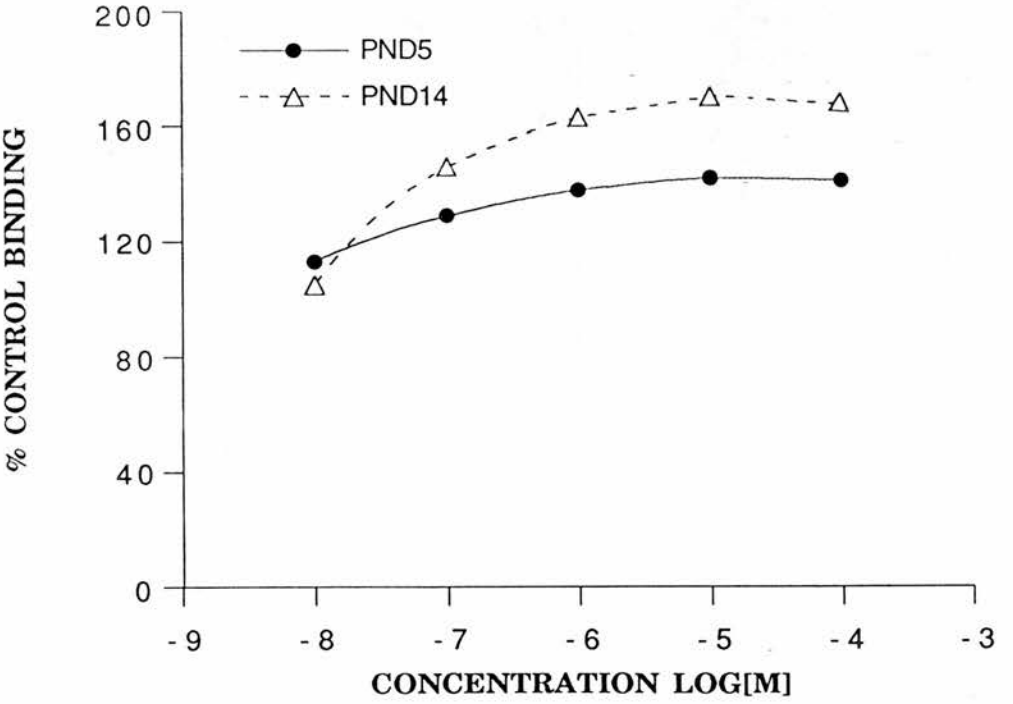


FIGURE 55 : Modulation of [^{3}H]dizocilpine binding to synaptosomal membranes at two postnatal ages

Binding of [^{3}H]dizocilpine was measured as previously described in Fig.44, but in the presence of incresasing concentrations of L-glutamate(A) or glycine(B: 0.01-100μM). Binding was expressed as a percentage of binding under control conditions.

A.EC_{50} values of 0.19μM and 0.22μM were calculated for L-glutamate modulation at PND5 andPND14 respectively.

B.EC_{50} values of 0.11μM and 0.10μM were calculated for glycine modulation at PND5 andPND14 respectively.

TABLE 14 EC_{50} VALUES FOR L-GLUTAMATE AND GLYCINE MODULATION OF [^{3}H]DIZOCILPINE BINDING TO SYNAPTOSOMAL MEMBRANES

Membranes were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the presence of increasing concentrations of L-glutamate (A) or glycine (B) (0.01 - 100μM). Non-specific binding was measured with dizocilpine (30μM). Data were fitted to the logistic equation $Y = MX^P/(X^P + EC_{50})$. Analysis of variance revealed no significant differences between ages.

A: L-Glutamate

AGE RANGE (days)	EC_{50} (μM)	n	MEAN AGE (days)
0 - 4	NM		
5 - 8	0.17 ± 0.05	5	6.4
9 - 12	0.10 ± 0.07	4	10.0
13 - 16	0.08 ± 0.06	4	14.5
17 - 20	0.08 ± 0.05	4	18.8
21 - 24	0.06 ± 0.01	3	23.3
25 - 28	0.06 ± 0.02	3	27.7
90	0.28 ± 0.05	15	90

B: Glycine

AGE RANGE (days)	EC_{50} (μM)	n	MEAN AGE (days)
0 - 4	NM		
5 - 8	0.09 ± 0.05	5	6.4
9 - 12	0.25 ± 0.09	4	10.0
13 - 16	0.07 ± 0.01	4	14.5
17 - 20	0.15 ± 0.03	4	18.8
21 - 24	0.05 ± 0.01	3	23.3
25 - 28	0.14 ± 0.07	3	27.7
90	0.11 ± 0.01	13	90

Values represent mean ± s.e.m.

NM: not measured

3.7.21 Alternative evaluation of EC_{50} variation for L-glutamate and glycine

Plots of all EC_{50} values for L-glutamate and glycine were made with respect to age and straight lines fitted as described previously (Fig.56). The line of best fit had a positive gradient for L-glutamate data. This may indicate a postnatal change. However the line of best fit for glycine data had a slope close to zero indicating a lack of postnatal change. Inclusion of PND90 data did not influence either line.

3.8 [^{3}H]CPP BINDING TO SYNAPTOSOMAL MEMBRANES THROUGHOUT POSTNATAL DEVELOPMENT

Synaptosomal membranes were prepared from pooled cerebral cortices and hippocampi as previously described and as used for [^{3}H]dizocilpine binding. This enables a direct comparison with [^{3}H]dizocilpine binding data.

3.8.1 Specific binding of [^{3}H]CPP

Specific binding was measured throughout postnatal development and as for [^{3}H]dizocilpine binding was divided into age ranges (Fig.57). [^{3}H]CPP binding was low at PND0-4 and increased gradually with age (Fig.57A). Binding at PND0-4 (mean age 3.6d; 9.88 ± 2.8 fmol/mg protein; n = 3) was not significantly different from that at PND5-8 (mean age 7.3d; 7.34 ± 1.8 fmol/mg protein; n = 3). It accounted for around 15% of the binding measured in adult rats (PND90; 49.5 ± 3.8 fmol/mg protein; n = 11). Binding increased from PND9-12 onwards, reaching around 50% of adult levels by PND13-16 (mean age 13.4d; 25.94 ± 4.6 fmol/mg protein; n = 4). Maximum [^{3}H]CPP binding was measured at PND21-24 (mean age 21.8d; 52.9 ± 3.2 fmol/mg protein; n = 3). This corresponds to 107% of the PND90 value. Analysis of variance revealed a significant difference between binding at different ages ($F(6) = 14.621$; $p < 0.01$). Only binding at PND21-24 was not significantly different from that at PND90. All other binding was significantly lower than at PND90 ($p < 0.05$).

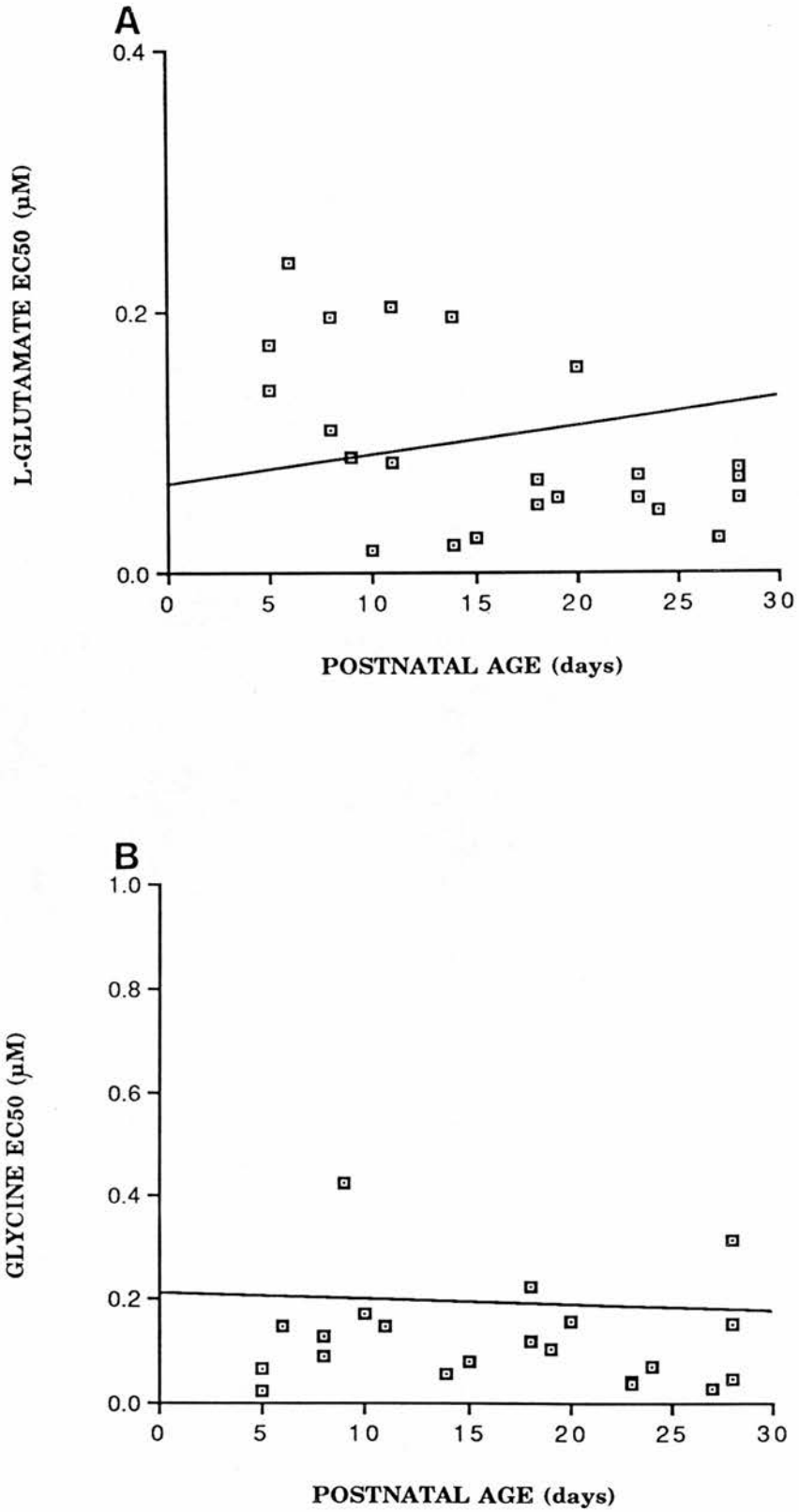


FIGURE 56: Alternative evaluation of the EC_{50} values for L-glutamate and glycine. 2
Individual EC_{50} values for L-glutamate(A) and glycine(B), have been plotted against postnatal age and fitted using the method of least squares with a straight line, using the equation; y=mx+c, to estimate the variance. L-Glutamate(A) modulation reveals a line with a positive gradient. This may indicate a postnatal change in the ability of L-glutamate to modulate binding. The line of best fit for glycine modulation has a gradient very close to zero indicating a lack of postnatal change.

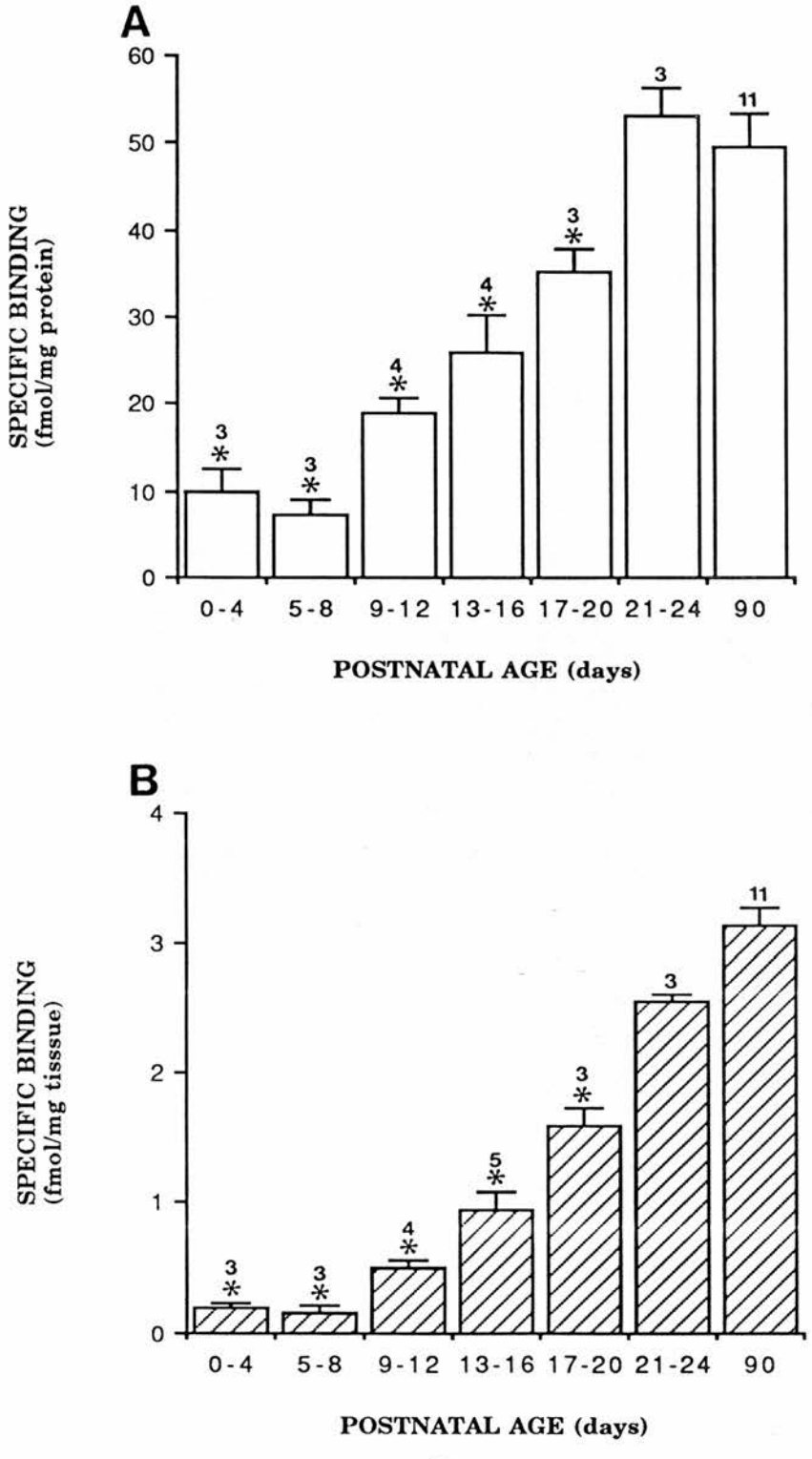


FIGURE 57: Postnatal binding of [^{3}H]CPP to synaptosomal membranes
Membrane samples were incubated at 25°C for 25min with[3H]CPP (10nM) in the absence or presence of L-glutamate (1mM) to measure total and non-specific binding. Specific binding was calculated at individual ages both per mg protein (A) and per mg wet weight tissue (B). Values were grouped and meaned as explained in the text. Statistical analysis was performed using one-way analysis of variance followed by a t-test for each set of data. A significant effect was seen when $p < 0.05$, see text for full details.
*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

When [^{3}H]CPP binding data was expressed per mg tissue a similar profile is seen (Fig.57B). Binding was again low throughout the first postnatal week followed by a gradual increase throughout postnatal development. At PND0-4 and PND5-8 the amount of binding is very low (0.193 ± 0.03 and 0.161 ± 0.05 fmol/mg tissue respectively; n = 3) being around 5% of the adult level. Levels of binding reach approximately 50% of the adult amount at PND17-20 (mean age 18d; 1.6 ± 0.13 fmol/mg tissue; n = 3) this is later than when expressed per mg protein. At PND21-24 (mean age 21.8d; 2.54 ± 0.07 fmol/mg tissue; n = 3) binding has reached just over 80% of the adult level. Significant differences identical to those seen when expressed per mg protein were found with analysis of variance, $F(6) = 16.336$; $p < 0.05$. Binding between PND0 and PND20 is significantly lower than at PND90 ($p < 0.05$).

3.8.2 Binding parameters for [^{3}H]CPP binding during postnatal development

As for [^{3}H]dizocilpine binding it is important to determine whether these observed changes in the amount of specific binding throughout postnatal development are due to changes in receptor affinity or to receptor density. Thus [^{3}H]CPP binding was inhibited with increasing concentrations of CPP over the range 0.03μM to 100μM. K_d and B_{max} values were calculated.

3.8.3 K_d for [^{3}H]CPP binding during postnatal development

K_d values for [^{3}H]CPP binding are displayed in Table 15. Between PND0 and PND8 specific [^{3}H]CPP binding was too low to give reproducible inhibition, therefore these K_d values could not be calculated. A K_d value of 0.39μM was however calculated in one instance at PND8. This value is similar to the adult value (PND90; 0.37 ± 0.06μM; n = 11). The K_d value for [^{3}H]CPP binding does not change throughout postnatal development. Thus, analysis of variance revealed no significant differences between K_d values calculated at different ages postnatally, $F(4) = 0.072$.

TABLE 15 K_d VALUES FOR [^{3}H]CPP BINDING

Membranes were incubated with [^{3}H]CPP (10nM) at 25^{o}C for 25 min in the presence of increasing concentrations of CPP (0.03 - 100μM). Non-specific binding was measured with L-glutamate (1mM). IC_{50} values were estimated graphically and K_d values calculated using Equation 2 (Section 2.7). No significant differences between ages were revealed with analysis of variance.

AGE RANGE (days)	K_d (μM)	n	MEAN AGE (days)
0 - 4	NM		
5 - 8	0.39	1	8.0
9 - 12	0.31 ± 0.13	3	10.7
13 - 16	0.48 ± 0.16	4	13.4
17 - 20	0.33 ± 0.10	3	18.0
21 - 24	0.36 ± 0.11	3	21.8
90	0.37 ± 0.06	11	90

Values represent mean ± s.e.m.
NM: not measured

Alternative evaluations of the variance in K_d are shown in Fig.58. Both plots (SB/B_{max} and all K_d values) have lines of best fit with gradients close to zero. This supports the findings of the analysis of variance.

3.8.4 B_{max} values for [^{3}H]CPP binding during postnatal development

Corresponding B_{max} values were calculated (Fig.59). The lowest B_{max} value was found at PND5-8 (PND8; 0.323pmol/mg protein; n = 1). The B_{max} values increased gradually from this age reaching a maximum value at PND90 (2.57 ± 0.49pmol/mg protein; n = 11). Analysis of variance revealed a significant difference between ages, $F(4) = 5.769$; $p < 0.05$. Further analysis revealed that B_{max} values between PND9 and PND16 were significantly lower than at PND90 ($p < 0.05$).

3.8.5 Comparison of B_{max} values for [^{3}H]CPP binding and [^{3}H]dizocilpine binding

[^{3}H]CPP B_{max} values were compared with those for [^{3}H]dizocilpine binding in the presence of L-glutamate and glycine to synaptosomal membranes. No differences were seen between the two ligands when corresponding ages were compared ($F(4) = 0.874$.

3.9 THE EFFECT OF AN ACUTE HYPOXIC INSULT ON THE BINDING OF [^{3}H]DIZOCILPINE TO PND0 CENTRAL TISSUE : PRELIMINARY DATA

The perinatal brain is particularly susceptible to neuronal damage via activation of the NMDA receptor following an hypoxic-ischaemic insult (Andine *et al*, 1988; McDonald *et al*, 1989). Having established the existence of the NMDA receptor from birth (PND0) onwards, a preliminary study was undertaken on PND0 Lister Hooded rat pups which had been subjected to an acute hypoxic insult in utero (Kendall *et al*, 1991). Tissue was obtained from control and hypoxic pups having been subjected to the

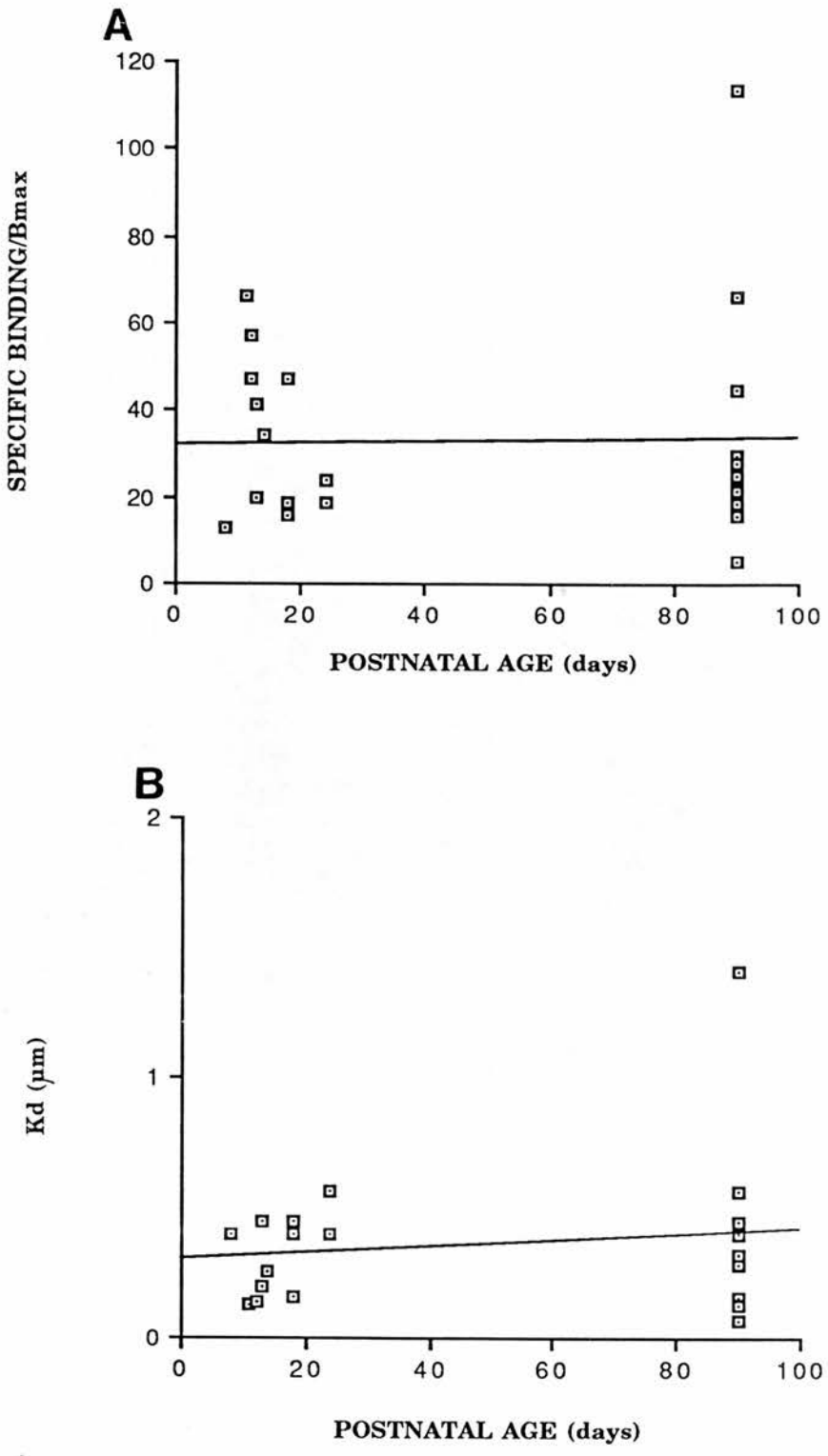


FIGURE 58: Alternative evaluation of the postnatal variance in the K_d for [^{3}H]CPP binding to synaptosomal membranes

Each measurement of K_d, Bmax, and specific binding (calculated per mg protein) has been used to estimate the postnatal variance in receptor affinity for [^{3}H]CPP binding. Straight lines(y=mx+c) have been fitted using the least squares method to plots of individual determinations of specific binding/Bmax (A) and K_d(B). The gradients of both lines is very close to zero, indicating a lack of change during postnatal development.

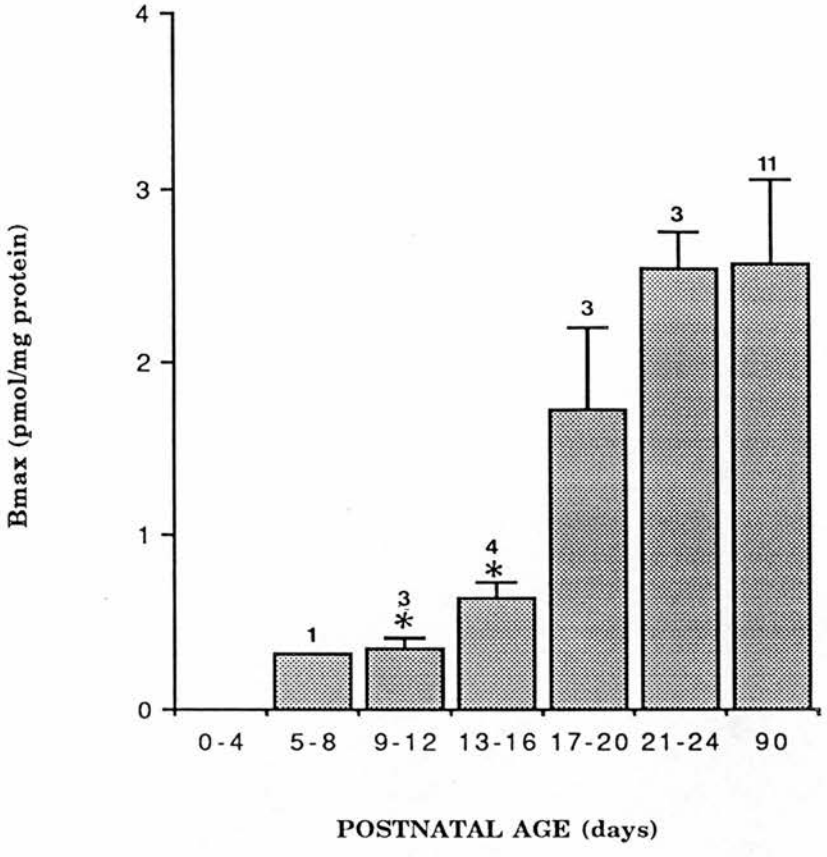


FIGURE 59: Postnatal Bmax values for [^{3}H]CPP binding

Bmax values were calculated as explained in Fig.54. Statistical analysis was performed using one-way analysis of variance followed by a t-test. A significant effect was seen when $p < 0.05$, see text for full details. Due to low specific binding, K_d and hence Bmax values could not be measured at PND0-4.

*, $P < 0.05$ when compared to PND90 value. Number of observations are indicated above each column.

procedure described in Appendix 1. A high mortality rate is the biggest problem associated with this procedure, consequently lack of viable tissue restricted these studies.

The first study compared [^{3}H]dizocilpine binding between control and hypoxic tissue with a comparison of the levels of binding in a cortical/hippocampal preparation and a cerebellar preparation. Whole membranes were used (Table 16). Binding, regardless of the method of expression was always greater in the cerebellum than the cortex/hippocampus. [^{3}H]Dizocilpine binding to cortical/hippocampal membranes from hypoxic pups could be modulated to a greater extent than the control membranes. However, lack of tissue precluded further studies to find out whether this is a significant effect. An interesting observation is that in both control and hypoxic tissue no increase in binding was observed in cerebellar membranes with L-glutamate and glycine. In each case a decrease in the amount of specific binding was observed. The percentage increase seen in membranes prepared from cortical/hippocampal tissue is very similar to that already described for whole membranes (Fig.36).

The data in Table 17 are for [^{3}H]dizocilpine binding to synaptosomal membranes prepared from control and hypoxic tissue. It appears that there is no change in the specific binding detected between control and hypoxic tissue, whether expressed per mg protein or mg tissue, with these values being very similar to those in Fig.44. Binding of [^{3}H]dizocilpine to both control and hypoxic tissue was modulated by both L-glutamate and glycine (10μM) as measured by an increase in specific binding. The major difference between hypoxic and control binding is that the percentage increase in binding due to L-glutamate/glycine modulation is less in the tissue from the hypoxic pups. However, further studies should be carried out to indicate whether this effect is significant. This may be an initial indication of an alteration in NMDA receptor function.

TABLE 16 BINDING OF [^{3}H]DIZOCILPINE TO CONTROL AND HYPOXIC TISSUE 1. WHOLE MEMBRANES

Membranes prepared from either pooled cortical and hippocampal tissue or cerebellar tissue were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the absence and presence (modulated) of L-glutamate and glycine (both 10μM). Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated and expressed as a percentage of control binding.

BINDING CONDITIONS	SPECIFIC BINDING fmol/mg protein	% CONTROL BINDING	SPECIFIC BINDING fmol/mg tissue	CONTROL % BINDING
HYPOXIC				
1. Cortex/hippocampus				
control	25.69	-	0.76	-
modulated	56.73	221	1.67	220
2. Cerebellum				
control	87.77	-	2.22	-
modulated	46.76	- 47	1.18	- 47
CONTROL				
1. Cortex/hippocampus				
control	30.33	-	0.84	-
modulated	47.50	157	1.32	157
2. Cerebellum				
control	127.66		2.9	-
modulated	76.01	- 39	1.77	- 39

Values are means of two independent observations.

TABLE 17 BINDING OF [^{3}H]DIZOCILPINE TO CONTROL AND HYPOXIC TISSUE 2. SYNAPTOSOMAL MEMBRANES

Membranes prepared from pooled cortical and hippocampal tissue were incubated with [^{3}H]dizocilpine (1nM) at 25°C for 45 min in the absence or presence (modulated) of L-glutamate and glycine (10μM). Non-specific binding was measured with dizocilpine (30μM). Specific binding was calculated and modulated binding expressed as a percentage of control binding.

BINDING CONDITIONS	SPECIFIC BINDING fmol/mg protein	% CONTROL BINDING	SPECIFIC BINDING fmol/mg tissue	% CONTROL BINDING
HYPOXIC				
control	15.59	-	0.09	-
modulated	49.16	315	0.28	311
CONTROL				
control	12.39	-	0.08	-
modulated	50.91	411	0.31	407

Values are means of two independent observations.

CHAPTER 4

DISCUSSION

4.1 INTRODUCTION

The pharmacology of the NMDA receptor has been extensively investigated. This is not only for experimental reasons but also because compounds acting as antagonists at this site have great potential as therapeutic compounds (Herrling, 1989). A great deal of research has been directed towards finding compounds active as antagonists at specific sites on the NMDA receptor and which cross the BBB easily while having minimum side effects. To date, the compounds described, are dizocilpine which binds to a site within the channel, competitive antagonists of the neurotransmitter site, antagonists of the strychinine insensitive glycine site and compounds acting at the polyamine site (Williams *et al*, 1991; Collingridge and Lester, 1989). The technique of radioligand binding has become an important tool in the study of drug/hormone receptor interactions. This is largely due to the increased availability of biologically active compounds radiolabelled to a high specific activity. The present study has investigated the NMDA receptor complex using this technique in both immature and mature brain tissue. Radioligands with high affinities for their binding sites were used. Generally, the higher the affinity of a radioligand the lower the associated non-specific binding, since lower ligand concentrations can be used. By referring to the criteria of Bylund and Yamamura (1990) the proportion of specific binding detected with [^{3}H]CPP and [^{3}H]D-AP5 (i.e. < 50%) is "barely adequate" while that seen with [^{3}H]dizocilpine (> 90%) is "excellent". Thus, the greater the proportion of specific binding, the greater the quality of the assay. This emphasises why it is important to establish optimal experimental conditions.

The reason for using the "barely adequate" ligands ([^{3}H]CPP and [^{3}H]D-AP5) is that they are very selective for their binding site on the NMDA receptor. Foster and Fagg (1987) compared and contrasted radioligands which bind to the NMDA neurotransmitter recognition site. They concluded that [^{3}H]L-glutamate was the best ligand since it had the highest proportion of specific binding associated with its binding. However since D-AP5 and NMDA could only inhibit 70-80% of binding, it was not

binding exclusively to the NMDA receptor. [^{3}H]NMDA and [^{3}H]D-AP5 although very selective were found to be least suitable since they were associated with very low specific binding (30-40%).

Only radiolabelled antagonists have been used in the current study. Radiolabelled agonists may label only a proportion of the total population of receptors (Thedinga *et al*, 1989). Radiolabelled antagonists on the other hand generally label the total receptor population (Bylund and Yamamura, 1990). The binding of radiolabelled agonists may however be a more accurate reflection of the physiologically relevant receptor population. This situation is particularly relevant to studies of the NMDA receptor where agonist (labelled by [^{3}H]L-glutamate) and antagonist (labelled by [^{3}H]CPP) states are proposed to exist (Monaghan *et al*, 1988). In this study evidence for heterogenous antagonist receptor states is suggested by the differential modulation of ligands binding to the neurotransmitter recognition site via the allosteric glycine modulatory site. Monaghan (1991) has provided evidence that [^{3}H]dizocilpine binding sites are also heterogeneous.

4.2 [^{3}H]CPP, A COMPETITIVE NMDA ANTAGONIST

CPP has been shown to be highly specific for the NMDA receptor by having no significant effects in around twenty other neurotransmitter binding assays (Lehmann *et al*, 1987) as well as other functional assays (Childs *et al*, 1988). However, a report appeared claiming that CPP inhibited the binding of [^{3}H]flunitrazepam having a K_i value of 430pM, making it 250 times more potent at the benzodiazepine site than at the NMDA receptor (White *et al*, 1988). This was later disproved by Williams *et al* (1988) who demonstrated reciprocally that neither CPP nor CGS19755 had any ability to displace [^{3}H]flunitrazepam binding to central membranes.

The optimal experimental conditions found for [^{3}H]CPP binding are in general agreement with those used by other research groups (Murphy *et al*, 1987; Pullan *et al*, 1990). Inclusion of Ca^{2+} (> 1mM) in the incubation buffer of radioligand binding assays has been reported to reduce the proportion of non-specific binding (Olverman *et*

al, 1988). However this was not found in the current study. The concentration of assay buffer can readily influence binding of radioligands (Wilkin *et al*, 1981; Scott *et al*, 1992). In the current study [^{3}H]dizocilpine binding was more easily measured using 5mM Tris-HCl than 50mM Tris-HCl. 5mM buffer proved unsuitable for measuring [^{3}H]CPP binding. This is most likely a membrane interaction rather than a receptor interaction since L-glutamate modulation of [^{3}H]dizocilpine binding can be measured with 5mM and 50mM buffer. This effect is mediated via the same site as [^{3}H]CPP is binding to. To eliminate any effects chloride ions (Tris-HCl) might be having on binding, Tris-acetate was substituted as assay buffer. No differences were observed, thus chloride ions were probably not influencing binding. Binding was highest at pH 7.75, this is in agreement with the findings of Murphy *et al* (1987) who demonstrated maximal [^{3}H]CPP binding at pH 7.6-7.8. Reduction of endogenous EAA's was attempted by extra washing of the membranes preparation. No improvements on binding were measured. This may reflect a low concentration of residual EAA's in the synaptosomal membrane preparation. This is further supported by the fact that [^{3}H]dizocilpine binding to an identical membrane preparation could be extensively modulated by L-glutamate and/or glycine (see Section 3.3). Under the conditions used in the present study [^{3}H]CPP bound with a high affinity to a single population of receptors which display similar affinities for the antagonist CPP and the agonist L-glutamate as previously reported (Watkins and Olverman, 1988; Murphy *et al*, 1987).

Like the present study, most other studies have restricted [^{3}H]CPP binding to synaptosomal preparations. Freshly prepared whole membranes were found to be unsuitable in this study due to low specific binding. However a couple of studies have successfully measured [^{3}H]CPP binding to whole membranes using a filtration method to terminate binding (Erdö and Wolff, 1990; Ogita and Yoneda, 1990b). Centrifugation is the preferred method of assay termination due to the rapid dissociation of [^{3}H]CPP from its binding site (Watkins and Olverman, 1988; Pullan *et al*, 1990; Murphy *et al*, 1987). Evidence that the use of a filtration assay for [^{3}H]CPP binding

may not result in the same population of sites being labelled as those in a centrifugation assay comes from the findings of Monaghan *et al* (1988). They described modulation of [^{3}H]CPP binding by glycine which was different to that seen in the present study and others (see Section 4.4; Olverman and Watkins, 1989). Termination of [^{3}H]CPP binding by filtration in this study resulted in very similar findings to that of Murphy *et al* (1987), i.e. very little specific binding was detected.

Ogita and Yoneda (1990b) not only used a filtration assay for termination of the binding process they also used membranes which were detergent treated (0.08% Triton X-100). They report that this increased the affinity of [^{3}H]CPP for its binding site three times to 0.081μM with no alteration in B_{max} value. This is the lowest K_d value reported for [^{3}H]CPP binding, and helps to explain why they could successfully use a filtration assay. Other studies employing detergent treated membranes do not report such high affinity values. Murphy *et al* (1987) do report an enhancement of specific binding in the presence of Triton compared with untreated membrane preparations. While the use of detergents in preparing membranes for use in ligand binding studies may be beneficial, problems do exist (Helenius and Simons, 1975).

4.3 [^{3}H]DIZOCILPINE, A NON-COMPETITIVE NMDA ANTAGONIST

Binding of [^{3}H]dizocilpine was measured in both whole and synaptosomal membrane preparations prepared from rat brain tissue. This ligand bound to a single population of binding sites in both membrane preparations. Binding could be inhibited by unlabelled dizocilpine and increased by the addition of L-glutamate and/or glycine. The extent of this modulation was clearly dependent on the concentration of these amino acids available at the receptor. This is consistent with the established use dependency of this compound (Davies *et al*, 1988).

The binding data presented here were best fitted to a single site for binding to both membrane preparations in agreement with other studies (Foster and Wong, 1987; Wong *et al*, 1986, 1988). Morin *et al* (1989) detected two [^{3}H]dizocilpine binding sites, a high affinity (K_d = 4nM) site and a low affinity (K_d = 110nM) site, in the rat

cortex. Other studies, have also detected two similar binding sites for [^{3}H]dizocilpine binding, however not after 45 min incubation as used in the current study and by Morin *et al* (1989). High and low affinity sites are detected after much longer incubation periods when binding has reached "true" equilibrium and modulation by L-glutamate and glycine cannot be detected (Javitt and Zukin, 1989). Javitt and Zukin (1989) propose that [^{3}H]dizocilpine binding reaches "true" equilibrium after four hours of incubation with membranes. After this time modulation by L-glutamate and glycine cannot be detected. They suggest that NMDA agonists act to increase the association rate but not the affinity of [^{3}H]dizocilpine binding. They propose a complex model of ligand, receptor interaction whereby dizocilpine can bind to both open and closed conformations of the NMDA receptor. Morin *et al* (1989) proposed that their high affinity site may represent the true NMDA receptor binding site while the low affinity site may be the PCP or σ site.

The binding of [^{3}H]dizocilpine to whole and synaptosomal membrane preparations, was variable under different experimental conditions. Buffer concentration may influence binding. Binding was more easily measured using 5mM Tris-HCl (pH 7.4) compared with 50mM Tris-HCl (pH 7.4). A similar effect of buffer on [^{3}H]dizocilpine binding has recently been reported (Scott *et al*, 1992). Buffer concentration has previously been demonstrated to influence specific binding of radioligands to identified neurotransmitter receptors (Wilkin *et al*, 1981). This is likely to be a non-specific membrane effect and not a receptor mediated phenomena. Tris buffer has been reported to have toxic metabolic effects on cells (Gillespie and McKnight, 1976). Bowery *et al* (1988) measured [^{3}H]dizocilpine binding autoradiographically using 5mM and 50mM Tris-HCl at physiological pH. They found no differences in binding. In the present study however it was decided that the lower buffer concentration provided optimal conditions for binding and that it would enable comparisons with other studies to be made more easily (Foster and Wong, 1987; Wong *et al*, 1988; Ransom and Stec, 1988).

The concentration of EAA agonists available at the NMDA receptor is an important consideration not only in functional but in radioligand binding studies. Most studies aim to reduce the endogenous EAA content of their membrane preparation such that exogenous modulation of [^{3}H]dizocilpine binding can be measured. In the present study this was done by extra washing and freeze/thawing of the membranes. The reduction in endogenous EAA's is reflected by the reduced affinity of [^{3}H]dizocilpine for its binding site. Extra washing led to an increase in B_{max}, the reason for this is unclear but extra preparation time may have lead to changes in the membranes such that important intracellular constituents were lost or adversely affected. Scott *et al* (1992) have reported that extensive washing and freezing of membrane preparations was deleterious to [^{3}H]dizocilpine binding. However in this study only extensive washing appeared to be deleterious to binding. An alternative method employed to reduce endogenous EAA's is to treat membranes with detergent (Ogita and Yoneda, 1989). Pharmacologically the [^{3}H]dizocilpine binding site in Triton treated membranes resembled the site described in the current study. The extent of modulation was not greatly different from that reported here and elsewhere (Foster and Wong, 1987). It can be assumed that such treatment provides no greater benefits than the methods used here to reduce endogenous EAA's.

Both L-glutamate and glycine enhanced [^{3}H]dizocilpine binding as described previously (Loo *et al*, 1986; Reynolds *et al*, 1987; Wong *et al*, 1988; Foster and Wong, 1987; Ogita and Yoneda, 1990a). The extent varied between membrane preparations, reflecting the endogenous EAA content. Least modulation was detected with fresh (normal washed) whole membranes, greatest modulation was seen with previously frozen synaptosomal membranes. These two preparations are assumed to have the highest and lowest endogenous EAA content. However, one notable effect was that glycine always modulated [^{3}H]dizocilpine binding to a lesser extent than L-glutamate. L-glutamate and glycine when used in combination increased binding to a greater extent than L-glutamate alone. This is consistent with other reports (Tremblay *et al*, 1990). To obtain a functional response to glycine an NMDA agonist must be

present (Johnston and Ascher, 1987). Since glycine alone has no effect (but see Meguro *et al*, 1992), it is proposed that the response to glycine is due to residual EAA's in the membrane preparations. The activation state of the NMDA receptor in a membrane preparation is therefore uncertain unless the concentration of EAA's is known. For this reason some [^{3}H]dizocilpine binding studies have used very crude unwashed membranes since the endogenous EAA concentration is assumed to be high enough to maximally activate all available NMDA receptors (Paleos *et al*, 1990; Majeswka *et al*, 1989). There are however many other endogenous factors which may be influencing the binding of dizocilpine including polyamines, Mg^{2+} and Zn^{2+} (Wong *et al*, 1988; Tremblay *et al*, 1990; Williams *et al*, 1991). It will prove difficult to regulate all of these factors experimentally. Consequently washing or freeze/thawing membranes will also alter the concentrations of these modulators, which may in turn influence the binding of site specific radioligands to the NMDA receptor.

Specific binding is higher in whole membrane preparations in the absence of added modulating amino acids than in synaptosomal membrane preparations. This is probably due to the higher endogenous EAA levels in whole membrane preparations which would explain the higher affinity of [^{3}H]dizocilpine for its binding site under control conditions (see Section 3.3.3). The increased binding in whole membranes is not due to an increased receptor density since the B_{max} for [^{3}H]dizocilpine binding is greater in synaptosomal membranes. Whole membrane preparations differ from synaptosomal preparations in that they contain membrane fragments from all the cell whereas synaptosomal membranes contain material concentrated from the neuronal synapse. Binding to receptor sites in synaptosomal membrane preparations is likely to represent physiologically relevant synaptic receptors. Binding to whole membrane preparations may include extra-synaptic receptors though in the case of the NMDA receptor no evidence for their existence is currently available (Erdö and Wolff, 1989). Usowicz *et al* (1989) have provided evidence for the existence of L-glutamate receptors on glia. However, on the particular astrocytes studied only non-NMDA responses could be detected. The magnitude of the response to L-glutamate was however

equivalent to that of NMDA in neuronal cells. The role of such EAA receptors is unclear (Gallo *et al*, 1989; Usowicz *et al*, 1989). It is possible that NMDA receptors may exist on glia. Both membrane preparations contain glia, and may therefore both allow binding to more than just the synaptically located, NMDA receptor.

The rank order of potency displayed by the stereoisomers and racemic mixture of dizocilpine differed between the two membrane preparations. This was maintained in the presence of L-glutamate. Monaghan (1991) proposed heterogeneous [^{3}H]dizocilpine sites. This may explain the current finding in that [^{3}H]dizocilpine is binding to a slightly different population of receptors in each membrane preparation. This however remains to be demonstrated.

Although dizocilpine has been demonstrated to block the NMDA associated ion channel, the physiological relevance of this binding site remains unclear. One possibility is the existence of an endogenous substance which acts to modulate NMDA receptor activity via the dizocilpine binding site. Zukin *et al* (1987) have presented evidence that such a substance exists in extracts of brain tissue. However it may be more specific for the PCP/σ site than the NMDA dizocilpine/PCP site. This would seem to warrant further research since the existence of such a substance may have clinical relevance.

4.4 INTERACTIONS BETWEEN THE NMDA NEUROTRANSMITTER SITE AND GLYCINE MODULATORY SITE

Glycine has been demonstrated here and elsewhere (Wong *et al*, 1988; Loo *et al*, 1986) to modulate the binding of [^{3}H]dizocilpine and [^{3}H]TCP to membrane preparations. The increase in specific binding measured here in the presence of glycine is due to an increase in dizocilpine binding site affinity. Functionally, glycine alone is inactive at the NMDA receptor (Nowak *et al*, 1984). The modulatory effect of glycine in [^{3}H]dizocilpine binding studies may be due to endogenous EAA agonists in the membrane preparation. This infers that a modulatory interaction takes place between the neurotransmitter site and the allosteric glycine on the NMDA receptor complex.

This interaction was investigated using [^{3}H]CPP and [^{3}H]D-AP5 binding and the results obtained provide further evidence for the differential modulation of the neurotransmitter recognition site via the glycine site, as originally demonstrated by Monaghan *et al* (1988). Alternatively the results may provide evidence for distinct isoforms of the NMDA receptor (Monaghan, 1991).

4.4.1 Glycine

Although endogenous glycine concentrations in the synaptosomal membrane preparations were not measured, using an identical membrane preparation to that used in [^{3}H]CPP and [^{3}H]D-AP5 binding studies, modulation of [^{3}H]dizocilpine binding by exogenously applied glycine could be measured. It is proposed that the glycine site is not fully activated under the experimental conditions employed in this study although it is likely to be partially activated due to residual endogenous glycine.

Glycine was demonstrated to modulate the binding of [^{3}H]CPP and [^{3}H]D-AP5. Assuming that it is acting through the same site, this is a further indication that the NMDA associated glycine site is not saturated in this membrane preparation. Dose-dependent enhancement and inhibition (± 20%) were seen for [^{3}H]CPP binding with maximal binding measured in the presence of 3μM glycine. Only a substantial dose-dependent inhibition (50%) was seen for [^{3}H]D-AP5 binding, with maximum inhibition measured in the presence of 3μM glycine. This suggests that a differential modulation of non-competitive antagonist binding by glycine exists. These two competitive antagonists were previously thought to bind to the same receptor site in a similar fashion (Watkins and Olverman, 1988). Thus two possibilities exist, binding may be to one site but in a different manner or else to two separate sites with which glycine interacts differently.

Olverman and Watkins (1989) have reported similar findings for [^{3}H]D-AP5 and [^{3}H]CPP binding, modulated with either D-serine or glycine. Fagg & Baud (1988) however reported that glycine at concentrations up to 100μM did not influence the binding of [^{3}H]CPP. These differential effects of glycine on [^{3}H]CPP binding may be

due to different concentrations of endogenous glycine in the membrane preparations. Monaghan *et al* (1988) conversely report an effect of glycine on [^{3}H]CPP binding that is identical to that seen on [^{3}H]D-AP5 binding in the present study (i.e. 50% inhibition). Danysz *et al* (1989) also demonstrated only inhibition (20%) of [^{3}H]CPP binding with increasing concentrations of glycine. These studies differ from the present one in that the binding assays were terminated using the filtration method. Filtration may result in a different population of NMDA receptors being labelled with [^{3}H]CPP to that labelled ina centrifugation assay. [^{3}H]CPP binding in a filtration assay may be to the same population or state of NMDA receptors as detected by [^{3}H]D-AP5 in a centrifugation assay. In a centrifugation assay [^{3}H]CPP is thought to bind to the agonist as well as the antagonist state of the receptor (Olverman and Watkins, 1989). Monaghan *et al* (1988) also reported an effect of glycine on NMDA sensitive [^{3}H]glutamate binding which resembled that of glycine on [^{3}H]CPP binding in the present study. This may support the theory that [^{3}H]CPP binds to both agonist and antagonist states of the receptor (Olverman and Watkins, 1989).

The main reason for the differences in binding between [^{3}H]D-AP5 and [^{3}H]CPP in the presence of glycine is thought to be their different structures. It is possible that the two compounds, CPP which contains 7 carbon atoms (C-7) and is conformationally fixed and, D-AP5 which contains 5 carbon atoms (C-5) and is conformationally flexible, are able to induce different conformational states of the receptor by binding in a differential manner. This may therefore allow glycine to differentially modulate the neurotransmitter binding site depending on the conformational state induced. Table 18 summarises the reported interactions between the glycine site and the neurotransmitter site.

[^{3}H]CGP 39653 is the most potent competitive NMDA antagonist yet synthesised (K_d = 6nM). The effect of glycine has now been investigated on [^{3}H]CGP 39653 (C-7) binding (Sills *et al*, 1991). Glycine inhibited 63% of the binding in a biphasic manner resulting in IC_{50} values of 0.54μM and 0.87μM. This is proposed to be allosteric modulation and not competitive inhibition (Sills *et al*, 1991). This is quite

TABLE 18 REPORTED INTERACTIONS BETWEEN THE NMDA NEUROTRANSMITTER SITE AND THE ASSOCIATED GLYCINE SITE

RADIOLIGAND	GLYCINE	HA-966	7-Clkyn
[^{3}H]CPP	↑↓(1, 2) ↓(3, 5) ↔(4)	↑(1, 3, 9)	↓(1, 3)
[^{3}H]D-AP5	↓(1, 2)	↓(1)	↓(1)
[^{3}H]L-Glutamate	↑↓(5)	↔(10)	↓(11)
[^{3}H]CGP 39653	↓(6)	↔(12)	

	CPP	HA-966	D-AP5	NMDA	L-GLUTAMATE
[^{3}H]Glycine	↔(7) ↓(8)	↓(10)	↓(7, 8)	↔(7)	↔(7)
[^{3}H]L-689,560	↓(7)		↓(7)	↑(7)	↔(7)

Abbreviations:

↑ Enhancement of binding

↓ Inhibition of binding

↑↓ Enhancement and inhibition of bindng

↔ No effect on binding

Numbers in parenthesis refer to the following references:-

1. Present Study
2. Olverman and Watkins (1989)
3. Danysz *et al* (1989)
4. Baud and Fagg (1988)
5. Monaghan *et al* (1988)
6. Sills *et al* (1991)
7. Grimwood *et al* (1991)
8. Monahan *et al* (1989)
9. Pullan *et al* (1990)
10. Foster and Kemp (1989)
11. Kemp *et al* (1988)
12. Pingping *et al* (1991)

different to the effect of glycine on [^{3}H]CPP binding (C-7). It would seem therefore that the differences in binding may be more subtle than just assuming the basic C-5, C-7 structural differences. Perhaps each of these different structures allows the NMDA receptor to adapt differing conformational states in the presence of glycine, and thus multiple states of the receptor can exist, or else multiple receptor subtypes exist each having differing sensitivities to competitive antagonists at the neurotransmitter binding site.

Reciprocal investigations of the effects of these competitive antagonists on [^{3}H]glycine binding have also been reported (Table 18). C-5 compounds (D-AP5, CGS-19755, CGP 37849) all decreased binding of [^{3}H]glycine by around 50%. C-7 compounds on the other hand had no effect or caused slight inhibition of [^{3}H]glycine binding (Monahan *et al*, 1990; Grimwood *et al*, 1991b). These findings would appear to agree with the effects of C-5 and C-7 compounds which would be predicted by the present study, although strictly speaking C-7 compounds may have been predicted to enhance [^{3}H]glycine binding. Endogenous glycine levels may have influenced the results.

Having demonstrated that glycine (3μM) maximally enhanced [^{3}H]CPP-binding, this concentration of glycine was investigated on the inhibition of [^{3}H]CPP binding and compared with inhibition under control conditions. Inhibition by CPP (C-7), L-glutamate (agonist) and 7-Clkyn (proposed to be an antagonist at the glycine site) was unaffected. Glycine is suggested to promote the agonist state of the receptor (Monaghan *et al*, 1988) [^{3}H]CPP is though to bind to both the agonist and antagonist states of the receptor (Olverman and Watkins, 1989). This may lead to the prediction that the effect of glycine would be to increase the affinity of L-glutamate and CPP for the [^{3}H]CPP binding site due to an increased proportion of agonist sites. This was not detected. The affinity of D-AP5 was however significantly decreased in the presence of glycine compared to control conditions, further confirming differential regulation of C-5 and C-7 antagonist binding by glycine. This does support the proposal that D-AP5 binds mainly to the antagonist state of the receptor. Glycine may therefore be

promoting a state of the receptor (agonist state) which is unfavourable to C-5 antagonists but favourable to C-7 antagonists (which may bind to both states). These findings support the proposal of Monahan *et al* (1990) who suggest a close functional relationship between the C-5 antagonist site and the glycine site on the NMDA receptor complex. 7-Clkyn inhibition of [^{3}H]CPP binding was also unaffected in the presence of glycine, it may directly interact preferentially with the agonist state of the receptor (see Section 4.4.3).

4.4.2 HA-966

HA-966 has been proposed to be either an antagonist or a partial agonist at the NMDA receptor glycine site (Fletcher and Lodge, 1988; Foster and Kemp, 1989). A functional characterisation cannot be made from the current binding studies, but two different effects of HA-966 were seen on [^{3}H]D-AP5 and [^{3}H]CPP binding. [^{3}H]D-AP5 binding was partially inhibited by increasing concentrations of HA-966 while [^{3}H]CPP binding was greatly enhanced in dose-dependent manner. The effect on [^{3}H]CPP binding has been demonstrated elsewhere using both centrifugation and filtration assays (Pullan *et al*, 1990; Danysz *et al*, 1989). HA-966 appears to promote a state of the receptor which is favourable to [^{3}H]CPP binding.

HA-966 was also investigated on the inhibition of [^{3}H]CPP binding. Different results were also found from those for glycine modulation. The affinities of CPP and 7-Clkyn were increased in the presence of HA-966 with that of D-AP5 being unaltered. The affinity of L-glutamate was reduced in the presence of HA-966. This provides further evidence that glycine and HA-966 are interacting with the proposed C-7 and C-5 states of the NMDA receptor differently. These findings may be accounted for by HA-966 reducing the ability of glycine to confer the agonist preferring state of the receptor, thus the affinity for L-glutamate is reduced while that for CPP is increased since it also binds to the antagonist state of the receptor. However, in these studies glycine itself did not increase the affinity of L-glutamate for the [^{3}H]CPP site and HA-966 did not increase the affinity of D-AP5. HA-966 may be relieving the effect of endogenous

glycine on [^{3}H]CPP binding thus promoting an antagonist state of the receptor. Kemp and Priestley (1991) in agreement, propose from electrophysiological studies, that HA-966 has a higher affinity for the antagonist state of the receptor. Since the affinity of D-AP5 was not increased in the presence of HA-966 and HA-966 inhibited [^{3}H]D-AP5 binding it seems that this C-5 antagonist may be binding to a different site or receptor state from [^{3}H]CPP (C-7) such that a number of antagonist states may exist.

HA-966 enhances the binding of a C-7 antagonist to the NMDA receptor. However, it reduces the binding of a C-5 antagonist in a similar way to glycine. This effect on [^{3}H]D-AP5 binding may suggest that HA-966 has agonist properties at the glycine site. HA-966 also increased the affinity of 7-Clkyn for the [^{3}H]CPP binding site. These two compounds are proposed to be acting at the same site. In the present study only high concentrations of 7-Clkyn (1mM) influenced [^{3}H]CPP and [^{3}H]D-AP5 binding (see Section 3.4.6). This is probably a direct interaction with the neurotransmitter site since lower concentrations (30μM) are effective at the NMDA associated glycine site (Kemp *et al*, 1988). In the presence of HA-966, 7-Clkyn binds preferentially to the same site as CPP as reflected by the increase in their respective affinities. Pullan *et al* (1990) and Danysz *et al* (1989) have demonstrated that the enhancement of [^{3}H]CPP binding by HA-966 can be competitively inhibited by both 7-Clkyn and glycine. This is an indication that all three compounds are acting at the same site. This is supported by the present study which demonstrated that increasing concentrations of glycine can inhibit the HA-966 enhanced component of [^{3}H]CPP binding with an IC_{50} of around 6μM.

Electrophysiologically HA-966 decreased the component of NMDA-mediated responses enhanced by glycine (Foster and Kemp, 1989) and reduced NMDA-mediated responses which could not be enhanced by glycine, in both cultured cells and more intact preparations (Fletcher and Lodge, 1989). This suggests that HA-966 is an antagonist of glycine responses. HA-966 however also displayed agonist activity by increasing NMDA-mediated responses when glycine concentrations were low (Foster and Kemp, 1989; Huettner, 1990; Henderson, *et al*, 1990). [^{3}H]Glycine binding is

inhibited by HA-966 in a competitive manner with variable IC_{50} values (Pullan *et al*, 1991; Foster and Kemp, 1989). Spermine increased the affinity of glycine agonists but not antagonists in [^{3}H]glycine binding studies (Pullan *et al*, 1991). Thus the affinity of HA-966 like that of glycine was increased while the affinities of 7-Clkyn and DNQX, antagonists at the same site, were unaffected. It is also possible that the glycine site itself exists in different conformational states perhaps conferred by different compounds leading to differential interactions with the different states of the neurotransmitter site. HA-966 also competitively antagonised the modulatory effect of glycine but not L-glutamate on [^{3}H]dizocilpine binding, resulting in displacement of the dose response curve to the right (Kloog *et al*, 1990). All these effects are overcome by increasing glycine concentrations. These properties have lead to the suggestion that HA-966 is most likely a partial agonist possessing a low intrinsic activity (Foster and Kemp, 1989).

HA-966 does not however display the same effects as glycine on [^{3}H]CPP binding, although a similar effect is seen on [^{3}H]D-AP5 binding. A possible explanation for this is that the conformational states induced by the different structures of CPP and D-AP5 are differentially modulated not only by glycine but also by HA-966, which is not chemically similar to glycine and may therefore interact with the glycine site in a different way. The differences seen in the present study, between the effects of HA-966 on C-5 and C-7 antagonist binding are, like the differential effects of glycine, further complicated by the effect of HA-966 on the binding of [^{3}H]CGP 39653 (C-7). Pingping *et al* (1991) report that HA-966 is without effect on the binding of this C-7 antagonist, but report a similar enhancement of [^{3}H]CPP binding as described here. They propose multiple NMDA neurotransmitter sites in the rat brain.

4.4.3 7-Chlorokynurenic Acid

7-Clkyn, has been demonstrated to have increased NMDA antagonist activity compared to kynurenate (a broad spectrum EAA antagonist) by selectively antagonising the effects of glycine at the strychnine insensitive glycine site (Kemp *et al*,

1988). However, 7-Clkyn has also been proposed as an inverse agonist at the glycine site (Kemp *et al*, 1988; Foster and Kemp, 1989). This study has demonstrated that 7-Clkyn has a different effect on [^{3}H]CPP binding from HA-966 and glycine. 7-Clkyn inhibited the binding of [^{3}H]CPP, in a dose-dependent manner, though inhibition was incomplete. [^{3}H]D-AP5 binding was also inhibited in an identical manner. The inhibition of [^{3}H]CPP and [^{3}H]D-AP5 may be due to a direct interaction with the neurotransmitter binding site, as already mentioned, since inhibition is only seen at high concentrations (K_i ~ 100μM). This is similar to the effect of 7-Clkyn on NMDA-sensitive [^{3}H]L-glutamate binding (Kemp *et al*, 1988). Lower concentrations of 7-Clkyn (30μM) are effective at blocking glycine responses mediated via the NMDA-associated glycine site (Kemp *et al*, 1988). In the two binding assays in the present study no effect of 7-Clkyn at 30μM was seen. These findings suggest that 7-Clkyn does not distinguish between C-5 and C-7 antagonist binding and since it also has a similar effect on [^{3}H]L-glutamate binding it may not preferentially bind to the agonist or antagonist state of the receptor.

In the presence of increasing glycine concentrations 7-Clkyn inhibited binding of [^{3}H]CPP to around 20% of maximal binding, as seen in the presence of 7-Clkyn (1mM) alone. This component of binding is therefore 7-Clkyn and glycine insensitive. This suggests that 7-Clkyn is not interacting with the glycine site in a competitive manner and thus a direct interaction with the [^{3}H]CPP site was occurring. In the presence of increasing concentrations of HA-966 however this component of binding remaining in the presence of 7-Clkyn (1mM) could be inhibited completely. HA-966 also increased the affinity of 7-Clkyn for the [^{3}H]CPP site. These findings may reveal a close interaction between the state of the [^{3}H]CPP site conferred by HA-966, and the favoured binding site of 7-Clkyn. Under control conditions (in the absence of modulators) 7-Clkyn does not appear to differentiate between C-7 and C-5 receptor states.

7-Clkyn inhibited [^{3}H]glycine binding dose-dependently (K_i = 0.56μM), being around eighty times more potent than kynurenate and having a greater affinity for the site than HA-966 (K_i = 14μM) (Kemp *et al*, 1988; Kloog *et al*, 1990). Since 7-Clkyn at low micromolar concentrations had no effect on either [^{3}H]CPP or [^{3}H]D-AP5 binding a direct interaction with the neurotransmitter site has almost certainly been studied here. [^{3}H]TCP binding enhanced by glycine, was competitively antagonised by both 7-Clkyn (10μM) and HA-966 acting at the NMDA-associated glycine site (Kloog *et al*, 1990). However 7-Clkyn also had a similar effect on L-glutamate enhanced [^{3}H]TCP binding. This effect is due to an action at the neurotransmitter site. This demonstrates the lack of specificity of 7-Clkyn and indicates mixed sites of actions.

Two glycine site antagonists have been described and radiolabelled. L-689,560 and 5,7-dichlorokynurenate (5,7-DCKA) are both specific for the NMDA glycine site (Grimwood *et al*, 1991b; Baron *et al*, 1991). Grimwood *et al* (1991b) have investigated the effects of C-5 and C-7 competitive antagonists in addition to NMDA and L-glutamate on the binding of [^{3}H]L-689,560 (Table 18). The C-5 antagonists inhibited binding by around 30% while C-7 antagonists inhibited binding to 50% of control levels. L-glutamate had no effect on binding while NMDA decreased it by 30%. From the current study it may have been predicted that C-5 and C-7 competitive antagonists would have a similar effect on [^{3}H]L-689,560 binding since 7-Clkyn does not distinguish between [^{3}H]CPP and [^{3}H]D-AP5 binding. Grimwood *et al* (1991b) suggested that C-5 and C-7 competitive antagonists may confer differential conformational states on the NMDA receptor which influence the binding of glycine site agonists and antagonists. However, it may be that the converse is also true.

If HA-966 and 7-ClKyn are both acting at the glycine site, as a partial agonist and an antagonist respectively, it should be possible to block the agonist activity of HA-966 with 7-Clkyn. Conversely it should be possible to overcome the block of NMDA

responses by 7-Clkyn with HA-966. This would not only prove that these two compounds are acting at the same receptor site but that they are classified correctly. This remains to be demonstrated.

Kemp and Priestley (1991) propose that since 7-Clkyn has little effect on the kinetics of L-glutamate-mediated responses, that it is a "neutral" antagonist at the glycine site. The data presented in this study would suggest that it is also an inhibitor of C-7 and C-5 radioligand binding at the neurotransmitter site. However, modulation may suggest that it acts preferentially at the antagonist state, if that is the state of the receptor conferred by HA-966.

4.4.4 Physiological relevance

If [^{3}H]CPP does bind to agonist preferring states of the NMDA receptor, as well as to antagonist preferring states, then the findings of this study would appear to support the existence of these two states. Glycine promotes the agonist state while HA-966 promotes the antagonist state. The findings however are also in support of the theory that C-5 and C-7 competitive antagonists at the NMDA receptor promote different conformational states of the receptor leading to differential modulation via the allosteric glycine site. 7-Clkyn in agreement with other studies, is interacting directly with the neurotransmitter site at high concentrations, and is not causing its effect via the glycine site.

Recent evidence, obtained with the C-7 antagonist [^{3}H]CGP 39653, lead us to believe that the actual situation is not as clear cut as either of these theories might suggest. The binding of this compound is modulated differently to that of [^{3}H]CPP and [^{3}H]D-AP5 by glycine and HA-966 (Sills *et al*, 1991; Pingping *et al*, 1991). This therefore leads to the possibility of the existence of multiple NMDA neurotransmitter sites. Each may have differing sensitivities to agonists and antagonists, and be modulated differently via the glycine site. It has recently been proposed that the NMDA glycine site may also be heterogeneous (Yoneda *et al*, 1991). This theory, like the others remains to be demonstrated, but may be resolved by receptor cloning studies

which are undoubtedly underway. They may lead to the discovery of multiple NMDA receptor subtypes as has already been demonstrated for other EAA receptors (Tanabe *et al*, 1992; Keinanen *et al*, 1990).

Monaghan (1991) has demonstrated differential modulation of [^{3}H]dizocilpine binding in the presence of saturating concentrations of spermine and glycine. The anatomical profile of [^{3}H]dizocilpine binding in the presence of low concentrations of L-glutamate (0.3μM) resembled the profile of [^{3}H]L-glutamate binding. In the presence of high concentrations of L-glutamate (10μM) the profile resembled that of [^{3}H]CPP binding. He proposed the existence of distinct isoforms of the NMDA receptor as opposed to differential states regulated by glycine. The cerebellar NMDA receptor has also been proposed to be different from other characterised NMDA receptors due to its different pharmacological profile (Monaghan, 1991).

The physiological significance of these findings is still unclear. These effects are all manifest by exogenous compounds (apart from glycine). It is unknown whether under normal physiological conditions, the NMDA receptor, and glycine site are going to exist in antagonist states, unless there is an endogenous substance to act on the site. However, it is likely that in pathological conditions, in which the NMDA receptor is implicated, that the interactions between the glycine site and the neurotransmitter site are altered in some way resulting in different proportions of receptor states. The existence of isoforms of the NMDA receptor with differential sensitivities to endogenous agonists and exogenous antagonists does seem a more likely proposal. Connick and Stone (1983) suggested that quinolinate may act a subpopulation of NMDA receptors. It is possible that the other proposed EAA neurotransmitters (see Section 1.2) may also act preferentially at different states or populations of NMDA receptors with different physiological consequences. In the future therefore it may be possible to target one population of NMDA receptors which are specifically responsible for certain physiological or pathological phenomenon.

As yet the role of glycine in higher brain regions is not as clearly defined as lower regions. Some studies have demonstrated modulation of NMDA responses by glycine while others demonstrate that glycine has no effect (Fletcher and Lodge, 1988; Kemp *et al*, 1989; Ascher and Johnston, 1989; Thomson *et al*, 1989; Salt, 1989). This may be due to different regional or synaptic levels of glycine. Likewise the mechanism responsible for controlling release of glycine, and the source of the glycine is still unknown. To further our understanding of NMDA receptor actions, the neurotransmitter role of glycine in higher brain structures needs to be further investigated.

4.5 ONTOGENY OF THE NMDA RECEPTOR IN RAT CNS

4.5.1 Introduction

The choice of a synaptosomal preparation for an ontogeny study may not be completely appropriate. This is because the density of synaptic junctions is low at early postnatal ages in rat CNS. However, such a preparation has been used successfully in other developmental studies (Tremblay *et al*, 1990; Dudek *et al*, 1989). Measurements of [^{3}H]CPP binding to whole membrane preparations, as commonly used in receptor ontogeny studies (Erdö and Wolff, 1990; Majewska *et al*, 1989; Paleos *et al*, 1990), was not possible therefore a synaptosomal preparation was used at all postnatal ages. This preparation also proved suitable for measuring [^{3}H]dizocilpine binding during postnatal development. Binding of [^{3}H]dizocilpine was also measured to whole membranes during postnatal development as a comparison.

Binding of both ligands to synaptosomal membranes and [^{3}H]dizocilpine to whole membranes suggests that the ion channel and neurotransmitter site are both present from an early stage of development. Binding of each ligand during postnatal development, was to the same population of binding sites labelled by [^{3}H]dizocilpine and [^{3}H]CPP in synaptic and whole membranes prepared from mature tissue. Binding of [^{3}H]dizocilpine to both membrane preparations could be modulated throughout

postnatal development by the amino acids L-glutamate and glycine. This modulation was as reported previously for binding of [^{3}H]dizocilpine and [^{3}H]TCP to adult tissue (Loo, 1986; Reynolds *et al*, 1987; Foster and Wong, 1987; Morin , 1989).

4.5.2 Postnatal development of the [^{3}H]dizocilpine binding site

Binding of [^{3}H]dizocilpine to synaptosomal and whole membranes was measured throughout postnatal development. This ligand bound to a single high affinity binding site corresponding to that previously characterised (Wong *et al*, 1986; 1988). Although it has around a 30-fold higher affinity for its binding site than [^{3}H]CPP for its binding site measurements of specific binding were still difficult to obtain from very young rats (PND0-4). This may be a reflection of the low density of binding sites at such an age. [^{3}H]CPP binding sites are also present in low numbers at this age. Since binding of [^{3}H]dizocilpine requires the presence of agonist, either residual endogenous EAA's or exogenously applied, it may be that reduced agonist binding site density reduces the probability of NMDA receptor activation to allow [^{3}H]dizocilpine access to its binding site. Binding of [^{3}H]dizocilpine to both membrane preparations was measured in the absence and presence of added L-glutamate and glycine. In the absence of added amino acids binding was probably due to activation of the NMDA receptor by residual amounts of endogenous EAA's. Nevertheless binding in the absence of added amino acids was very low, especially in the synaptosomal preparation. This may be a reflection of the lower levels of endogenous amino acids in the synaptosomal preparation and thus why a greater degree of modulation was seen upon addition of exogenous amino acids. Addition of L-glutamate (10μM) and glycine (10μM) maximally enhanced binding of [^{3}H]dizocilpine throughout postnatal development in agreement with other studies (Morin *et al*, 1989; Boje and Skolnick, 1992).

In the absence of added amino acids adult levels of binding to the whole and the synaptosomal membrane preparations were not reached at the same age range regardless of whether data were expressed per mg protein or per mg tissue. Adult

levels of binding are defined as the first age range when binding does not differ significantly from that at PND90. Binding reached adult levels at PND5-8 in synaptosomal membranes and PND13-16 in whole membranes when expressed per mg protein and at PND9-12 and PND13-16 respectively when expressed per mg tissue (Table 19). Levels of binding tend to rise to levels above that seen at PND90 before falling to the adult level. Binding profiles in the presence of L-glutamate or glycine largely mirror those of control binding in each membrane preparation. This suggests that no underlying modulatory mechanism is changing postnatally. Likewise the ages when adult levels of binding were reached mirror the control situation apart from binding to synaptosomal membranes in the presence of both amino acids, when binding reaches adult levels at a later age (PND9-12). This may be a reflection of the varying extent of modulation measured in the presence of both L-glutamate and glycine in this preparation throughout postnatal development. The reason for this is unclear since the extent of modulation by individual amino acids did not vary postnatally. This may be a reflection of an alteration in neurotransmitter site/allosteric glycine site coupling during postnatal development. Similar alterations in extent of modulation of [^{3}H]dizocilpine binding were detected with whole membranes. When expressed per mg protein specific binding reached adult levels at the same age as in the presence of the individual amino acids. Specific binding expressed per mg tissue reaches adult levels at later ages than when expressed per mg protein. The pattern of ages when adult levels are reached under each modulatory condition is the same for whole and synaptosomal membranes although the actual ages differ between preparations. The differences between data expressed per mg protein and per mg tissue are not uncommon in receptor ontogeny studies (Baudry *et al*, 1981; Sanderson *et al*, 1982), and serves to illustrate the importance of expressing binding data using both methods.

Adult binding is therefore consistently reached at a later age in whole membranes. These differences may be due to differences between the constituents of the membrane preparations. Whole membrane preparations will contain both synaptic and non synaptic material. The differences in the development profiles may be due to

TABLE 19 AGE RANGES WHEN AMOUNTS OF [^{3}H]DIZOCILPINE AND [^{3}H]CPP BINDING AND B_{max} VALUES FIRST REACH A LEVEL NOT SIGNIFICANTLY DIFFERENT FROM THAT SEEN AT PND90

EXPERIMENTAL CONDITION	SPECIFIC BINDING		B_{max}
	mg protein	mg tissue	
A. [^{3}H]DIZOCILPINE			
1. WHOLE MEMBRANES			
control	13 - 16	13 - 16	5 - 8
+ L-Glutamate (10μM)	13 - 16	17 - 20	5 - 8
+ Glycine (10μM)	13 - 16	17 - 20	9 - 12
+ L-Glutamate and glycine (both 10μM)	13 - 16	21 - 24	5 - 8
2. SYNAPTOSOMAL MEMBRANES			
control	5 - 8	9 - 12	5 - 8
+ L-Glutamate (10μM)	5 - 8	13 - 16	13 - 16
+ Glycine (10μM)	5 - 8	13 - 16	5 - 8
+ L-Glutamate and glycine (both 10μM)	9 - 12	90	5 - 8
B. [^{3}H]CPP			
normal conditions	21 - 24	21 - 24	17 - 20

Values represent age ranges as used previously in PND. Age ranges were determined by applying one-way analysis of variance followed by a t-test. They represent the age range when specific binding or B_{max} first reach a level not significantly different from that measured at PND90.

the incorporation in the whole membrane profile of binding to extra-junctional NMDA receptors. It is possible that if extra-junctional receptors exist they may follow a slightly different postnatal developmental profile from the receptor located in the synaptic cleft. The protein content of the different membrane preparations may be influencing the data since synaptosomal protein will be a subcomponent of that measured with whole membranes.

Binding expressed per mg protein and per mg tissue have distinct profiles presumably due to the postnatal alteration in protein content. If receptor number increases at the same rate as total protein content, no increase in binding would be seen postnatally. Since an increase in binding is seen postnatally, binding sites must be synthesised at a faster rate relative to total protein production. The rate of binding site synthesis must be maximal at the ages when transient peaks in binding the observed. In the current study transient peaks in [^{3}H]dizocilpine binding are seen from the third postnatal week. The subsequent decrease in binding to adult levels may represent a time when the rate of receptor synthesis relative to total protein synthesis is slowing down, thus the ratio of receptors to amount of protein will decrease. It has been suggested that the gradual reduction in the ratio of binding sites to total protein concentration partly reflects glial cell maturation which begins during the second postnatal week (Baudry *et al*, 1981).

The reason for the overshoot of adult levels of binding, which is quite common in receptor ontogeny is not fully understood. Baudry and Lynch (1982) and Tremblay *et al* (1988) suggest that it may be during a period when increased synaptogenesis is taking place. This may be particularly relevant since the NMDA receptor is implicated in CNS neuronal development (see Section 4.5.6; Hunt and Patel, 1990).

Changes in postnatal [^{3}H]dizocilpine binding would appear to be due to increased receptor density, rather than to increased receptor affinity. The postnatal increase in receptor density under each modulatory condition follows a similar profile to that seen for specific binding under the same condition. B_{max} is consistently higher at all ages in a synaptosomal preparation as would be predicted. B_{max} values reach

levels not significantly different from that seen at PND90, at the same age or earlier than levels of binding reach PND90 levels. The similar profiles for specific binding and B_{max} supports the finding that K_d is not altering postnatally.

L-Glutamate and glycine, separately or in combination increase specific binding as a result of an increase in receptor affinity. The rank order of potency is the same in immature and adult preparations. Throughout postnatal development the following order is seen, L-glutamate and glycine > L-glutamate > glycine. This is in agreement with previous reports (Tremblay *et al*, 1991; Foster and Wong, 1987). The extent of modulation by these amino acids is different in the two membrane preparations probably, as discussed earlier, reflecting the different endogenous EAA content. Generally, for each condition the extent of this modulation was similar at all ages to that seen at PND90. There were a couple of exceptions. L-Glutamate did not increase specific binding of [^{3}H]dizocilpine to whole membranes at PND0-4 compared to control binding. The corresponding K_d for binding at this age was higher than at all others. Therefore it may be that L-glutamate has a reduced ability to modulate [^{3}H]dizocilpine binding at this age. However, a combination of L-glutamate and glycine did increase specific binding. This finding at PND0-4 may well be due to the unreliability of the assay on membranes prepared from very young animals due to the low density of binding sites. The other differences were seen for binding in the presence of both amino acids to whole and synaptosomal membranes, when modulation at certain ages was significantly less than at PND90. Since modulation by individual amino acids was constant postnatally, and the postnatal EC_{50} values did vary significantly from that at PND90 this is an unusual finding. It may reflect postnatal alterations in the coupling between the neurotransmitter site and the allosteric glycine site.

Up to four hypotheses have been proposed as to why NMDA agonists increase the binding of [^{3}H]dizocilpine and [^{3}H]TCP. The first theory is that the increase in binding of these radioligands is due to an increase in receptor density with no alteration in receptor affinity (Javitt and Zukin, 1989). The developmental study of Tremblay *et*

al (1990) presented further evidence in support of this (see Section: Ontogeny of [^{3}H]TCP binding). They also demonstrated that B_{max} for [^{3}H]TCP binding increased in the presence of NMDA compared with binding in the absence of NMDA at PND9 with no alteration in K_d compared with control conditions suggesting that this mechanism is present postnatally. However, the findings of the present study suggest that at a given age the alteration in [^{3}H]dizocilpine binding in the presence of NMDA agonists is due to a change in affinity with no change in receptor density. This is in agreement with a number of other studies (Foster and Wong, 1987; Morin *et al*, 1989; Ransom and Stec, 1988; Reynolds *et al*, 1987). This is true at all postnatal ages, no matter the modulatory condition. Javitt and Zukin (1989), however go on to further their hypothesis by proposing that [^{3}H]dizocilpine interacts with multiple states of the NMDA receptor complex. The B_{max} is thought to increase because dizocilpine becomes trapped in the channel due to the presence of agonist. The final theory is that NMDA agonists increase the association and dissociation rates of the binding of [^{3}H]TCP again with no alteration in K_d. These increases in [^{3}H]TCP binding can only be measured in non-equilibrium conditions (Jaritt and Zukin, 1989; Bonhaus *et al*, 1989). They therefore suggest that at "true" equilibrium (4 hours of incubation) the K_d cannot be altered. This would therefore suggest that binding in the present study has not been performed at true equilibrium, although it has the advantage of being able to measure modulation of [^{3}H]dizocilpine binding. These conditions have been employed elsewhere for this reason (Boje and Skolnick, 1992). It must be concluded from these multiple findings and theories that the exact mechanism by which NMDA agonists modulate the binding of the non-competitive antagonists is still not fully understood.

Morin *et al* (1989) used [^{3}H]dizocilpine to study the development of the NMDA associated ion channel and present similar findings to those reported here. Using a well-washed (eight times after freeze/thawing) whole membrane preparation they measured [^{3}H]dizocilpine binding to three brain areas, the cortex, hippocampus and brainstem. The hippocampus showed highest levels of binding at all ages tested. In

both cortex and hippocampus [^{3}H]dizocilpine binding increased with increasing postnatal age. Interestingly binding to the cerebellum was relatively high at PND3 (twice the adult value) peaking at four times the adult value at PND15. This high binding may explain why in the present study binding to the cerebellum at PND0 was much higher than in the hippocampal/cortical preparation (Table 16). The cerebellar NMDA receptor is reported to be a different isoform to that found in higher CNS regions (Monaghan *et al*, 1991). This may explain these findings, in that it may have a developmental profile distinct from NMDA receptor(s) in higher CNS regions. A postnatal autoradiographical study of [^{3}H]dizocilpine binding would provide information about regional variations in the development, of [^{3}H]dizocilpine binding sites and would complement membrane binding studies. Unfortunately Morin *et al* (1989) do not study any ages after PND20 when it is possible that levels of binding overshooting the adult levels may have been detected thus corresponding with the data presented here. They do report that changes in [^{3}H]dizocilpine binding postnatally are due to alterations in receptor number and not affinity. Morin *et al* (1989) also measured the extent of the modulation of [^{3}H]dizocilpine binding in the presence of exogenous amino acids. In agreement with the present study they find no significant differences between modulation at PND7 and adult. Overall their extent of modulation is similar to that found here in whole membranes. Since they use a well washed (8 x) preparation it may have been expected that they would obtain a far greater degree of modulation due to negligible endogenous ligands (Foster and Wong, 1987). Morin *et al* (1989) do not quote the levels of endogenous neurotransmitter in their preparation, but they do find K_d values very similar to those found in this study.

Boje and Skolnick (1992) have investigated the effect of glycine on [^{3}H]dizocilpine binding during postnatal development to a whole membrane preparation. They too present similar findings to those found here in that the EC_{50} for glycine does not alter postnatally and that the alteration in postnatal specific binding is due to an alteration in receptor density rather than to an alteration in receptor affinity.

They report that adult levels of binding are reached at PND12. This age corresponds to that found in the present study. In addition they report that association and dissociation rates of [^{3}H]dizocilpine for its binding site do not alter postnatally.

Ontogeny of [^{3}H]TCP binding

Other studies relevant to this [^{3}H]dizocilpine ontogeny study are studies of the ontogeny of the [^{3}H]TCP binding site. [^{3}H]TCP binds to the dizocilpine site (Maragos *et al*, 1988; Wong *et al*, 1988). However, 60 - 80% of [^{3}H]TCP binding can be displaced by dizocilpine. The remaining 20% - 40% is almost certainly bound to another site, the σ site being the main candidate (Sonders *et al*, 1988). Therefore [^{3}H]TCP binding as a measure of the ontogeny of the dizocilpine binding site may not be equivalent to [^{3}H]dizocilpine binding, but may in fact incorporate binding to another site.

Two developmental studies have used [^{3}H]TCP to measure NMDA receptor binding to whole membranes (Paleos *et al*, 1990; Majewska *et al*, 1989) containing high concentrations of endogenous amino acids to allow maximal modulation. A further study has used well washed synaptic membranes (Tremblay *et al*, 1990). All three studies found an increase in the amount of specific binding with increasing age. Majewska *et al* (1989) present a very similar developmental profile to that presented here for [^{3}H]dizocilpine binding to whole membranes. Adult levels of binding were reached at PND14. They also demonstrate that binding gradually declines between PND90 to PND360. Paleos *et al* (1990) present a binding profile where adult values are reached at PND21, later than found by Majewska *et al* (1989). Both studies also characterise the ontogeny of the σ receptor using different ligands, showing that it has a postnatal developmental profile distinct from the NMDA ion-channel site.

Tremblay *et al* (1990) using synaptosomal membranes found adult levels of [^{3}H]TCP binding at around the third postnatal week (PND15-21) under control and modulated binding conditions. This is later than reported in the present study for [^{3}H]dizocilpine binding. Unlike this study postnatal binding never rose above adult

levels. They also examined the effects of NMDA and/or glycine as well as Mg^{2+} on [^{3}H]TCP binding throughout development. In agreement with this study they found modulation to be apparent at all ages tested. The extent of modulation also did not alter postnatally. Interestingly Mg^{2+} decreased [^{3}H]TCP binding from PND6 onwards. At this age endogenous Mg^{2+} block of NMDA mediated responses is not apparent (Bowe and Nadler, 1991). Tremblay *et al* (1990) also conclude that the changes seen in postnatal binding of [^{3}H]TCP are due to alterations in B_{max} but not to K_d throughout postnatal development. However, they demonstrate that NMDA agonists increase [^{3}H]TCP binding by increasing B_{max}, with no alteration in K_d during postnatal development. Their study also incorporated autoradiographical measures of [^{3}H]TCP binding, in the developing hippocampus showing similar profiles for the CA1 and CA3 regions. The CA1 region had consistently higher binding postnatally. [^{3}H]Dizocilpine has been successfully used previously in autoradiographical studies with mature tissue to examine regional binding (Bowery *et al*, 1987; Monaghan, 1991). As already mentioned an autoradiographical study using [^{3}H]dizocilpine in immature tissue has as yet not been reported. A study of this type would complement membrane binding studies. NMDA receptor isoforms have been proposed on the basis of differential [^{3}H]dizocilpine binding (Monaghan *et al*, 1991). A postnatal autoradiographical study, apart from providing information on sites in distinct and specific CNS regions, may provide information on the postnatal development of distinct receptor subtypes.

Ontogeny of the NMDA-associated glycine site

The developmental profile of the NMDA associated glycine site has been studied. Tremblay *et al* (1990) have characterised the site in the hippocampus using strychnine insensitive [^{3}H]glycine autoradiography. They showed that binding increased postnatally in all areas reaching adult levels by PND10. The postnatal distribution of binding was almost identical to that seen in adult tissue (Bristow et al, 1986), and also corresponded well with the distribution of [^{3}H]TCP binding sites

(Tremblay *et al*, 1990). Shinohara *et al* (1990) measured [^{3}H]glycine binding. They found a very similar development profile in rat forebrain membranes and in agreement with Tremblay *et al* (1990) demonstrated that this was due to an increase in receptor number and not to a change in receptor affinity. This supports the finding of the present study in that the EC_{50} for glycine did not alter postnatally, since these effects are manifest via the same site on the NDMA receptor complex.

4.5.3 Postnatal development of the [^{3}H]CPP binding site

[^{3}H]CPP binding during postnatal development increased gradually from low levels at PND0-4 with adult levels reached during the fourth postnatal week (PND21-24). The changes in the amount of specific binding were due to an increase in receptor density with increasing age. The affinity of the receptor for [^{3}H]CPP, did not alter postnatally, thereby suggesting that this is fixed during development. K_d values for [^{3}H]CPP binding measured postnatally, correspond to those previously reported for [^{3}H]CPP binding measured in adult tissue using a centrifugation assay (Watkins and Olverman, 1988; Lehmann *et al*, 1987). A similar developmental profile was found regardless of whether data was expressed per mg protein or per mg tissue. The [^{3}H]CPP binding site was expressed in neuronal membranes at a faster rate than total protein synthesis, since an increase in binding was seen with age. This profile, i.e. low binding at birth, reaching adult levels during PNW4, corresponds to the developmental profile of synaptic junction density as well as to the L-glutamate/aspartate and quinolinate concentration profiles in rat CNS (Erdö and Wolff, 1990b; Aghajanian and Bloom, 1967; Moroni *et al*, 1984b).

There has only been one other report in which [^{3}H]CPP has been used to study the ontogeny of the NMDA receptor. Binding of [^{3}H]CPP was measured to whole membranes prepared from rat visual cortex using a filtration assay (Erdö and Wolff, 1990a). They produced a binding profile almost identical to that produced in the present study, whereby binding was initially very low increased rapidly during the second post-natal week before reaching maximum binding levels around the fourth

postnatal week, before declining gradually until PND360. They likewise found the differences in the amount of binding between ages to be due to a change in receptor density rather than to a change in receptor affinity in agreement with the present study. However, the affinity value measured in the study of Erdö and Wolff (1990) was considerably higher than that measured here, and in other studies using a centrifugation assay (Olverman *et al*, 1986; Lehmann *et al*, 1987; Watkins and Olverman, 1988). It is possible that Erdö and Wolff are therefore not measuring the same state of the receptor as measured here using a centrifugation assay (Olverman and Watkins, 1989). No ontogenic studies have yet been reported using radiolabelled competitive NMDA antagonists which have a greater affinity for the binding site than [^{3}H]CPP, e.g. CPP-ene or CGS 39653 (Aebisher *et al*, 1989; Sills *et al*, 1991). Therefore the other studies with which this work must be compared are those using the less specific ligand [^{3}H]glutamate. However [^{3}H]CPP and [^{3}H]glutamate may be labelling different sites or different conformations of the NMDA site, the so called antagonist and agonist preferring sites respectively (Monaghan *et al*, 1988) or different receptor isoforms (Monaghan, 1991). It is possible that this could lead to different developmental profiles being measured.

Postnatal binding of [^{3}H]L-glutamate

Early studies of the ontogeny of EAA receptors using [^{3}H]glutamate binding did not account for the existence of EAA receptor subtypes. This therefore makes it very difficult to compare the data presented in the current study with that previously reported. Baudry *et al* (1981) and Sanderson and Murphy (1982) showed two different [^{3}H]glutamate binding profiles in the hippocampus and cortex respectively, during postnatal development. Both studies observed transient peaks in binding during postnatal development at PND9. These studies conclude that adult levels of binding are reached at PND23 in the rat hippocampus and at PND50 in the cortex. Adult binding at PND23 is in agreement with that found here for [^{3}H]CPP binding, although PND50 is considerably later. Likewise [^{3}H]glutamate binding to cerebellar tissue

showed a transient peak in the amount of specific binding, but at PND21 (de Barry *et al*, 1980). The data presented in the present study reveal no such pronounced transient peaks in binding regardless of the method of data expression, this may be because the membranes incorporate both cortices and hippocampi and thus transient binding, if site specific, is masked. Perhaps more likely it is because these early studies are measuring binding to multiple glutamate receptor subtypes. Erdö and Wolff (1990) also did not detect any postnatal transient peaks in binding with [^{3}H]CPP but did find them for both [^{3}H]KA and [^{3}H]AMPA binding during postnatal binding.

It is very difficult to adequately compare these three early reports since they all report different K_d values for L-glutamate and use different membrane preparations and assay conditions. Since a proportion of the Cl^-/Ca^{2+} independent [^{3}H]glutamate binding sites have been classed as potentially representing the NMDA receptor (Foster and Fagg, 1984) only the study of Sanderson and Murphy (1982) may be relevant to NMDA receptor ontogeny. However, their profiles bear least resemblance to those presented here or to that of Erdö and Wolff (1990).

NMDA sensitive [^{3}H]glutamate binding during postnatal development has since been measured. The results presented here are however not all in agreement with either of these studies (Tremblay *et al*, 1988; Represa *et al*, 1989). They reveal a transient increase in NMDA sensitive [^{3}H]glutamate binding sites during development, of the human and rat hippocampus using both radioligand binding and autoradiography. Transient peaks were detected at PND8 and foetal weeks 23-27 in rat and human tissue respectively. This transient peak in binding in the rat hippocampus would appear to occur at the same age as that found by Baudry *et al* (1982). In fact Tremblay *et al* (1988) make such a comparison but this is not an entirely valid comparison since Baudry *et al* (1982) only see their transient peak when expressing their data per mg protein and not when expressing their data per mg tissue. Tremblay *et al* (1988) do however find that adult levels of NMDA sensitive [^{3}H]glutamate binding are seen at PND22. This is in agreement with the data presented in this study and also that of Erdö and Wolff (1990). One finding which seems to be constant throughout all these studies

and in agreement with the present one is that the changes in postnatal binding are due to changes in B_{max} and not to alterations in K_d. This lack of change in affinity for [^{3}H]L-glutamate binding postnatally would agree with the lack of change in the EC_{50} for L-glutamate modulation of [^{3}H]dizocilpine binding as demonstrated here, since these effects are mediated via the same site on the NMDA receptor.

In contrast to the evidence for an increase in binding to NMDA sites being due to an increase in B_{max}, Garcia-Ladona *et al* (1990) present data which may suggest that in mouse deep cerebellar nuclei binding to EAA receptors remains constant postnatally. They tentatively suggest that their [^{3}H]glutamate binding paradigm may be labelling NMDA receptors, and that the B_{max} remains constant throughout postnatal development. This may be further evidence that the cerebellar binding site is different to that in higher brain regions, and as such has a different developmental profile.

One factor which seems to be constant throughout most of these studies and is also apparent from the data presented here, is that the NMDA binding site appears very early in development. Since it is present at PND0 it is almost certainly present prenatally. Represa *et al* (1989) provides evidence for this using human foetal tissue. They measured NMDA binding sites at 18 weeks of gestation. Binding at earlier ages have not been reported. In order to ascertain the significance of these binding sites at such an early stage of development, functional studies should be performed if at all possible to investigate the actions of the receptor at such an early stage of development. Such studies should also determine whether the characteristics of this prenatally expressed receptor are equivalent to those of the mature form of the NMDA receptor.

Insel *et al* (1990) using autoradiography performed the first detailed study of NMDA receptor ontogeny in the rat forebrain. NMDA sensitive [^{3}H]glutamate binding sites were measured. Highest levels of binding were seen in the CA1 region of the hippocampus, the dentate gyrus and the striatum at all ages studied (PND0-PND60), in agreement with a previous study using adult tissue (Monaghan *et al*, 1983). In all areas of the cortex and hippocampus binding increased postnatally to levels significantly higher than adult between PND14 and PND28 before decreasing to

PND60 levels. Interestingly at PND1 levels in the cortex and hippocampus ranged from 50% to 125% of adult levels. This is far higher than that seen with any radioligand binding study, including the present one. Insel *et al* (1990) report that a pattern of binding consistent with that seen in the adult becomes apparent from PND14. This study will be measuring the "agonist-preferring" state of the receptor as opposed to the "antagonist" state as measured here. This may therefore account for the different profiles and levels of binding. Miyoshi *et al* (1991) have performed [^{3}H]CPP autoradiography in the aged rat brain from two months to twenty one months of age. They find that binding decreases with increasing age to levels significantly lower than at two months of age in the cortex, caudate putamen and nucleus accumbens but not in the hippocampus (Miyoshi *et al*, 1990). This provides further evidence for site-specific alterations in NMDA binding sites during development.

A drawback of the data presented in the present developmental study therefore is that the membrane preparation consisted of two brain structures which may have different postnatal binding profiles. This preparation was used in an effort to try and facilitate the [^{3}H]CPP assay since binding is not particularly easy to perform, even using mature tissue. With the advent of the new radioligand [^{3}H]CGP 39653 (Sills *et al*, 1991) it should now prove easier to carry out such a study since the K_d for this compound is 6nM and the assay has the added benefit of using the filtration technique, thus making it easier to perform. This assay may measure the "antagonist preferring" state of the NMDA binding site and may be an interesting comparison to that of Insel *et al* (1990) who have measured the agonist state of the receptor. However, as already discussed [^{3}H]CGP 39653 may not be binding to the same receptor subtype or state as [^{3}H]CPP (see Section 4..4; Pingping *et al*, 1991). A different postnatal binding profile to that seen with [^{3}H]CPP may be found.

4.5.4 Comparison of [^{3}H]dizocilpine and [^{3}H]CPP postnatal binding

Adult levels of [^{3}H]dizocilpine binding (expressed per mg protein) regardless of the membrane preparation used and the modulatory condition employed, are reached at an earlier age than adult levels of [^{3}H]CPP binding in the current study. This is in agreement with many of the comparative studies (Tremblay *et al*, 1990; Erdö and Wolff, 1990a; Morin *et al*, 1989; Paleos *et al*, 1990). All the studies are in agreement that the postnatal changes in binding are due to an increase in receptor density with no corresponding changes in receptor affinity. The developmental profile of the NMDA glycine site most closely parallels that of the [^{3}H]dizocilpine site in the present study. This suggests that the rate limiting factor in NMDA receptor complex development may be the postnatal appearance of the neurotransmitter. The NMDA neurotransmitter site therefore develops at a slower rate than the ion channel site. One reason may be to limit the number of binding sites and hence to limit overactivation during early postnatal development when the immature CNS is susceptible to excitotoxic damage (McDonald *et al*, 1988)). Another possibility is to reduce the rate of synaptogenesis thus limiting the rate of programmed cell-death early in life.

The ratio of [^{3}H]CPP binding sites to [^{3}H]dizocilpine sites in synaptosomal membranes prepared from adult tissue is approximately 1:1 in the present study. This is in agreement with the findings of Thedinga *et al* (1989). Thedinga *et al* (1989) go on to suggest that the real ratio of NMDA neurotransmitter sites to ion channels is 2:1. This is because [^{3}H]CPP is only labelling one population of sites, the "antagonist-preferring" site and the density of NMDA-sensitive [^{3}H]glutamate sites must be taken into account. The differential rate of development of the two binding sites means that this ratio is going to alter during postnatal development probably with regional differences. An interesting reciprocal study which may provide a link between postnatal [^{3}H]CPP and [^{3}H]dizocilpine binding would be to investigate the effects of CPP, on [^{3}H]dizocilpine binding in the presence and absence of L-glutamate. The effects found should correlate well with the binding of [^{3}H]CPP to the same membrane preparation.

Since both L-glutamate and glycine, both separately and together, can modulate the binding of [^{3}H]dizocilpine to membrane preparations from very young animals these three sites must be functionally coupled from a very early stage of development, thus promoting NMDA receptor activation.

4.5.5 The postnatal development of non-NMDA receptors

The ontogeny of other EAA receptors has been studied. Two studies have examined the ontogeny of the metabotropic receptor (Palmer *et al*, 1988; Dudek *et al*, 1989). Metabotropic receptor linked IP metabolism was investigated postnatally in various brain regions. Different profiles were seen in the areas studied but generally in all areas IP stimulation was highest in the first postnatal week, decreasing with increasing age apart from in the olfactory bulb when a transient peak was seen at PND6 after which levels remained low until adulthood. Other notable transient peaks were observed in the hippocampus at PND9 and the cerebellum at PND6 (Palmer *et al*, 1988). During these transient peaks QA stimulation was also sensitive to modulation by NMDA indicating a mixed receptor population. This may correlate with the early peaks in NMDA sensitive [^{3}H]L-glutamate binding shown by Tremblay *et al* (1988) but it in no way reflects the data presented here for postnatal binding to the NMDA receptor. The findings may be an indication of the involvement of both the metabotropic and the NMDA receptor in synaptic development and maintaining synaptic plasticity in the developing CNS (Dudek *et al*, 1988; Nicoletti *et al*, 1986; Bear *et al*, 1987). The metabotropic receptor may have an important role to play in synapse development in the neonate, while NMDA receptor number is still low.

The KA receptor is the best studied non-NMDA receptor in the immature CNS. This receptor has been reported to increase in number with no apparent change in affinity, during postnatal development, in the cerebellum and hippocampus with peak levels reached during adulthood in rats and at birth in humans (Campochiaro and Coyle, 1978; Ben-Ari *et al*, 1984; Represa *et al*, 1986). Erdö and Wolff (1990a) found a very different pattern for both KA and AMPA receptor ontogeny. The density of both non-

NMDA binding sites in rat cortex peaked transiently at very high levels during the first few days of life, declining to adult levels, which were significantly lower than at birth, during PNW3-4. These transient peaks were reached at an even earlier age than those previously seen for NMDA-sensitive [^{3}H]glutamate binding (Tremblay *et al*, 1988). Postnatal autoradiographical studies of [^{3}H]KA and [^{3}H]AMPA binding (Miller *et al*, 1990; Insel *et al*, 1990) do not agree with the findings of Erdö and Wolff (1990). These studies indicate an increase in binding to both KA and AMPA sites with increasing age. Peak levels of [^{3}H]KA and [^{3}H]AMPA binding, higher than adult levels, were seen during PNW3-4. Another anomaly between these two sets of data concerns [^{3}H]AMPA binding. Erdö and Wolff (1990a) found the alteration in binding to be due to a change in receptor affinity with age whereas Insel *et al* (1990) proposed that their changes were due solely to an increase in receptor density. This is the only study of the postnatal development of EAA receptors where the affinity is proposed to alter with age. L-AP4, quisqualate, AMPA and ibotenate sensitive [^{3}H]glutamate sites have been demonstrated to be present in high density in the deep cerebellar nuclei of mice at PND10 decreasing in number by PND25 and remaining low in the adult (Garcia-Ladona *et al*, 1990). This is similar to their findings for the NMDA receptor. The significance of these results is unclear, although it may be an indication that different brain areas have different non-NMDA receptor ontogeny profiles as well as different NMDA receptor profiles (Insel *et al*, 1990; Miller *et al*, 1990).

From the diversity of studies on EAA receptor ontogeny it is difficult to adequately compare studies between different research groups and present a definitive picture of the postnatal development EAA receptors. Different ligands, membrane preparations, assay procedures and data presentation make comparisons difficult. In general a comparison of the present data for [^{3}H]CPP binding with that for the ontogeny of KA and AMPA receptors of Insel *et al* (1990) and Miller *et al* (1990) suggests that these receptors may develop in parallel. Perinatally [^{3}H]CPP binding sites, KA and AMPA sites are low although not in the study of Erdö and Wolff. A rapid increase in the number of binding sites seems to occur during the second postnatal

week, reaching or overshooting adult levels during postnatal weeks 3-4 before falling to adult levels. The [^{3}H]dizocilpine site appears to have a distinct developmental profile when compared to other EAA receptors. The postnatal profile of KA and AMPA receptor development found by Erdö and Wolff (1990a) would tend to confound the idea that these three receptor sites develop in parallel.

4.5.6 The relationship of postnatal binding to functional studies

Apart from the good correlation of NMDA receptor development with synaptogenesis and brain excitatory amino acid content, these data can be compared with findings from postnatal investigations of EAA receptors acquired using different techniques. The data from the present study shall be discussed in the context of electrophysiological studies, neurotrophic studies, neurotoxicity studies and concluding with a discussion on the role of NMDA antagonists.

(i) Electrophysiological studies

Murphy and Baraban (1990) have demonstrated in cultured ED17 (embryonicday) cortical cells that NMDA induced currents are very small until after 14-15 days in culture (suggested to correlate to PND8-9). A reason for small currents at these ages may be that there are few NMDA binding sites present and available to be activated. This would agree with the current findings for [^{3}H]CPP binding which is very low up to PND8. Of course there is the anomaly of culturing to be taken into account since there is no reason to believe that cultured cells behave as cells developing *in vivo* would.

The AP5-blockable component of L-glutamate induced currents has been examined in the rat visual cortex during postnatal development until PND21 (Stern and Sakmann, 1990). They demonstrated an increase in this component until PND21, however no adult data were reported in comparison. This study gives no indication of receptor number or affinity, but could be explained by an increase in receptor number as described here.

(ii) Postnatal neurotoxicity of EAA's

The toxicity of NMDA and other neurotoxins active at EAA receptors alters during postnatal development. KA is relatively non-toxic in the immature brain but is a potent neurotoxin in the adult brain reaching such levels at PND21 (Coyle, 1983). AMPA and QA both show a transient peak as neurotoxins between PND7 and PND14, with AMPA being up to 15 times more potent than QA (McDonald and Johnston, 1990a). L-Glutamate also leads to potent neurotoxic effects in the foetal brain when administered to pregnant mice and monkeys (Toth *et al*, 1987).

NMDA shows a well defined neurotoxic profile postnatally. This has been widely investigated in the developing rat by McDonald, Johnston and co-workers. Direct administration of NMDA (25nmol/0.5µl) into the striatum or the hippocampus at PND7 resulted in a lesion, 21 times and 16 times respectively, larger than the corresponding adult lesion. Damage at PND1, 14, 21 and 28 is of a similar degree to that seen in the adult. Intermediate damage is seen at PND4 and 10 (McDonald *et al*, 1988). Using a PND7 rat pup model of maximal NMDA toxicity the neuroprotective effects of competitive, non-competitive and glycine site neuroprotector, antagonists have been demonstrated, with dizocilpine being the most effective (McDonald and Johnston, 1990a, 1990b; McDonald *et al*, 1990a). All were effective after systemic administration, presumably since the blood brain barrier is not fully formed. Silverstein *et al* (1987) has also demonstrated that when rat pups (PND7) suffered an acute hypoxic-ischaemic insult this resulted in a reduction in [^{3}H]-glutamate binding thereby implicating EAA receptors in the resultant neurotoxic damage.

The present study does not show maximal binding to the NMDA neurotransmitter site at PND7. In fact quite the reverse is demonstrated since at PND7 there is no measurable increase in affinity for [^{3}H]CPP binding and no transient peaks in binding site densities. In addition, [^{3}H]dizocilpine modulation by L-glutamate and/or glycine is not enhanced at this age, with the EC_{50} values for modulation not showing any increase in potency. Apart from the few studies showing a transient expression of receptors at early ages which have not been reliably reproduced, receptor

number and increased receptor affinity would not seem to account for the increased toxicity of NMDA seen at this early age. However, adult levels of [^{3}H]dizocilpine binding are reached at PND5-8 in synaptosomal membranes, suggesting the ion channel is well developed, therefore Ca^{2+} will be able to easily enter cells. The lack of Mg^{2+} block, of NMDA responses at early postnatal ages, and lack of established inhibition will however allow the receptor to be activated more easily at PND7 thus allowing Ca^{2+} easy access to the cells. These effects are not however confined solely to PND7 (Morrisset *et al*, 1990; Bowe and Nadler, 1990; Ben-Ari *et al*, 1990). Further studies are required to establish the actual mechanism(s) of neurotoxicity at this particular age. The mechanisms regulating events occurring after NMDA receptor activation such as phosphorylation, protease, phospholipase and phosphatase activation which are all initiated upon Ca^{2+} influx during the cascade of events leading to eventual cell death (Choi, 1990) are likely to all have individual ontogenic profiles. One or more of these systems may be maximally expressed at PND7.

Quinolinate an NMDA agonist, does not show the same pattern of neurotoxicity in the immature brain as NMDA (McDonald and Johnstone, 1990a). This may be because quinolinate toxicity is mediated via a different pathway to that of NMDA neurotoxicity. Quinolinate is a week neurotoxin in the immature brain. Alternatively, this may be further evidence in support of the theory, that NMDA receptor subtypes exist with different developmental profiles and properties.

The preliminary data presented in this study demonstrated that following an acute hypoxic-insult *in utero*, at ED22/PND0, there is no immediate alteration in the binding of [^{3}H]dizocilpine, or of its ability to be modulated by L-glutamate and glycine. This binding was performed immediately after the insult (i.e. membrane preparation began within one hour of the insult). This is probably not enough time for EAA mediated damage to be manifest, but it is still evidence to support the hypothesis that the perinatal CNS is relatively resistant to damage produced by excessive EAA receptor stimulation during a hypoxic-ischaemic insult (Kendall *et al*, 1991; Cherici *et al*, 1991). The finding that binding remains unaltered at this age is in agreement with

biochemical and histological studies performed on tissue obtained at the same time point. At one hour post-insult the perinatal rat brain shows little signs of having suffered an hypoxic-ischaemic insult. Damage to the hindbrain however can be detected histologically after three days survival (Kendall *et al*, 1991). Due to the extreme difficulties in maintaining a sufficient number of control and hypoxic rat pups for any length of time postnatally, further information at present is limited. Binding studies would ideally be performed on pups from this model at various postnatal time points, including PND7 and adulthood to discover any postnatal changes in NMDA receptors which are manifest, as a result of a hypoxic insult over longer time periods. Autoradiography with competitive, non-competitive and glycine site ligands may prove to be more productive in this case since discrete alterations in NMDA receptor sites in isolated nuclei could be examined.

(iii) Neurotrophic role of EAA's

The NMDA receptor has been implicated as having an important role in promoting the survival of developing cells (Balazs *et al*, 1988a). NMDA (35μM) added to rat cerebellar granule cells (PND7 - 8) cultured in K^+ (5-15mM) prevented nerve cell loss normally seen in the presence of low K^+ concentrations. AP5, AP7 and dizocilpine all blocked this survival effect. AP5 had no effect on control cultures grown in the presence of 25mM K^+ and the absence of NMDA. Non-NMDA agonists had no effect on the low K^+ cultures (5-15mM). They concluded that chronic exposure of NMDA can promote the survival of nerve cells due to activation of the NMDA receptor. The extent of the effect was dependent on the concentration of NMDA and K^+ present and the length of time in culture (Balazs *et al*, 1988a,b; 1989). This neurotrophic effect involving the activation of the NMDA receptor suggests that exogenously released L-glutamate acting at the NMDA may be important for cell survival during development (Balazs *et al*, 1989). Similar effects were observed in cultured hippocampal granule cells (Brewer and Cotman, 1989). NMDA (20μM) and

L-glutamate (25μM) were both capable of increasing neurite branching and extension. Dizocilpine (20μM) blocked these effects thereby implicating the importance of NMDA receptor activation.

The NMDA receptor therefore has an important role in the development of the CNS. This explains why receptors are detected at such an early stage of life. Receptor number, as measured by [^{3}H]CPP and [^{3}H]dizocilpine binding, may be low to reduce excessive synaptogenesis during early life which would be wasteful of valuable resources in the foetus and neonate, or to limit neurotoxicity which may occur due to overactivation of the NMDA receptor.

A further reason for low receptor number in early life is that endogenous L-glutamate is also low (Erdö and Wolff, 1990b). Gelbard *et al* (1990) showed that depletion of dopamine (DA) in neonatal rats resulted in a significant loss of D_1 receptors. They suggest that in the absence of endogenous neurotransmitter receptors fail to develop adequately. Similar studies to establish the role of endogenous L-glutamate on receptor development would prove very difficult given the ubiquitous role of this neurotransmitter.

4.5.7 Cloning of the NMDA receptor

Electrophysiological evidence suggests functional differences between adult and immature NMDA receptors which may not be identified using radioligand binding or autoradiography. Cloning the adult and immature receptor, and determining their respective amino acid structures may reveal differential subunit structures between the two receptors as has previously been demonstrated for the muscle nicotinic acetylcholine (nACh) receptor (Mishina *et al*, 1986).

They demonstrated that the adult nACh receptor had the subunit composition α_2 ß ε δ whereas the immature receptor from foetal muscle had the sununit composition α_2 ß γ δ. Before innervation, the foetal receptor predominates, during synapse formation both receptors co-exist until the foetal receptor is fully replaced by the adult receptor during later synapse formation (Sakmann and Brenner, 1978). It is certainly possible

1978). It is certainly possible therefore that the NMDA receptor could be analogous to the nACh receptor since they are both linked to cation conducting ion channels and that functionally the NMDA receptor seems to alter postnatally.

Recently a number of reports have appeared describing the cloning of the adult NMDA receptor. Moriyoshi *et al* (1991) have cloned a single protein with physiological and pharmacological properties of the characterised NMDA receptor-channel complex. Meguro *et al* (1992) have cloned a different protein, which in combination with that of Moriyoshi *et al* (1991) results in a functional heteromeric NMDA receptor. These proteins show considerable homology with each other and also with cloned non-NMDA ionotropic receptors (Hollman *et al*, 1989; Keinanen *et al*, 1990). A detailed functional analysis of cloned adult and immature NMDA receptors may provide information on the nature of receptors from mature and immature tissue. In addition the existence of receptor subtypes during postnatal development may be demonstrated.

4.5.8 Therapeutic potential of NMDA antagonists in the immature brain

The presence of NMDA receptors early in life has been demonstrated. This may provide the opportunity to use NMDA antagonists therapeutically in disorders resulting from NMDA receptor activation in the neonate (McDonald and Johnstone, 1990a). The problem with this is undoubtedly the potential hazardous side effects. Acute therapy may be acceptable since the short-term side effects may be masked by the greater neuroprotective benefit to the patient. Side effects include depression of synaptic transmission and a transient interference with synaptic development, as well as a retardation of learning and memory mechanisms. There is evidence that acute administration of dizocilpine, as a neuroprotectant, may in fact lead to greater neuronal damage due to NMDA receptor upregulation, a particular hazard in the developing brain (McDonald *et al*, 1990b; McDonald and Johnston, 1990a). The potential for chronic therapy with NMDA antagonists may be extremely limited during postnatal

development again due to long term interference with synaptogenesis and the resultant adverse effects on CNS development, learning and memory. Therefore the risks of chronic treatment may outweigh any potential benefits.

4.6 CONCLUDING REMARKS

The main findings from this study are summarised below:-

1. [^{3}H]CPP and [^{3}H]dizocilpine each bind with high affinity to a single population of sites in immature and mature rat brain, the NMDA neurotransmitter site and the ion channel site respectively.
2. Glycine, a full agonist, and HA-966, a partial agonist at the NMDA glycine site discriminate between the binding of [^{3}H]CPP and [^{3}H]D-AP5. These two competitive antagonists were previously thought to be binding in a similar manner to the NMDA neurotransmitter site. HA-966 enhances the binding of [^{3}H]CPP while it inhibits the binding of [^{3}H]D-AP5. Glycine enhances and inhibits the binding of [^{3}H]CPP while it only inhibits the binding of [^{3}H]D-AP5.
3. 7-Chlorokynurenate, an antagonist at the glycine site on the NMDA receptor, does not differentiate between the binding of [^{3}H]CPP and [^{3}H]D-AP5. It weakly inhibits the binding of both ligands, probably through a direct interaction at the neurotransmitter binding site and not by an allosteric action at the glycine site. Evidence from 7-Clkyn actions in the presence of HA-966 however suggest that 7-Clkyn may bind preferentially to the state of the neurotransmitter site induced by CPP.
4. It is suggested that either [^{3}H]CPP and [^{3}H]D-AP5 are binding differently to the neurotransmitter site and thus inducing different conformational states of the receptor which are modulated differently by HA-966 and glycine, or binding of glycine and HA-966 may be inducing different conformational states of the glycine site which allow differential binding of ligands to the neurotransmitter site. These findings could also be explained by the existence of multiple NMDA receptor isoforms as opposed to one receptor in multiple conformations.

5. A synaptosomal membrane preparation has been successfully used in an ontogeny study to demonstrate the postnatal development of the NMDA neurotransmitter site and associated ion channel.

6. The binding of [^{3}H]CPP and [^{3}H]dizocilpine during postnatal development show different profiles. Adult levels of [^{3}H]dizocilpine binding are reached at an earlier age (PND5-8) than [^{3}H]CPP binding in an identical membrane preparation (PND21-24).

7. The differences between [^{3}H]dizocilpine binding to whole and synaptosomal membranes postnatally may be due to different populations of receptors being measured. Extra-synaptic receptors if they exist may also be measured using whole membranes.

8. The alterations in all postnatal binding were due to a change in receptor number rather than to an alteration in receptor affinity.

9. Modulation of [^{3}H]dizocilpine binding during postnatal development (PND0-PND28) by glycine and L-glutamate was similar to that seen in adult tissue. This suggests that the neurotransmitter site, allosteric glycine site and the ion channel on the NMDA receptor complex are functionally coupled from an early stage of development.

Molecular cloning of the NMDA receptor is currently underway. This may provide evidence for the existence of multiple NMDA receptor subtypes thus explaining the differences between [^{3}H]CPP and [^{3}H]D-AP5 binding. In addition, the structure of the immature NMDA receptor may be revealed to be distinct from that of the adult. This may explain why mature and immature receptors have different functional characteristics.

In summary, therefore NMDA receptors are present during development and antagonists of sites on the complex may have a potential as therapeutic agents for early postnatal ages. Acute administration may be ethically acceptable if the potential benefits outweigh the risks. Chronic therapy with such compounds is likely to be unacceptable during prenatal and postnatal development throughout childhood due to

the risks of harmful effects on CNS development outweighing any benefits. Further research is required in order to try and reduce the risks. Different subtypes of NMDA receptors may have different physiological roles. For example, NMDA receptor subtypes may be amenable to selective targeting, i.e. a selective "quinolinate receptor" antagonist may have different properties to NMDA antagonists already characterised. With the availability of so many sites for therapeutic targeting on the NMDA receptor it may be prudent to investigate the role of the different sites in NMDA receptor activation in various pathological states. Site-specific NMDA antagonists may therefore be useful therapeutically in the treatment of immature seizures, epilepsy and febrile convulsions, damage due to hypoxic-ischaemic insults, and amino acid metabolism disorders, non-ketotic hyperglycinaemia and sulfite oxidase deficiency which ultimately lead to brain damage in the infant due to overactivation of EAA receptors (McDonald and Johnston, 1990a). Investigation into the events occurring after NMDA receptor activation, and Ca^{2+} entry, which lead to neurotoxicity and eventual cell death may prove fruitful in that selective enzyme inhibition may result in fewer side-effects in the immature and mature brain.

APPENDIX 1: SURGICAL PROCEDURE TO INDUCE AN ACUTE HYPOXIC INSULT IN UTERO

Surgery was performed on time mated pregnant Lister Hooded rats. Rats were used 22 days after pregnancy was confirmed (ED22/PND0). Dams were anaesthetised using 2.5% isoflurane in a 30% O_2; 70% N_2O mixture, anaesthesia being maintained, throughout surgery, with 0.5 - 1% isoflurane.

A midline incision was made to expose one uterine horn. Each utero-placental blood vessel was clamped with artery clips and replaced in the dam for 30 min. At the end of the hypoxic episode the artery clips were removed and reperfusion took place. Pups were rapidly excised, the brains being used for biochemical and histological studies. Brain tissue used for these binding studies was obtained from pups not required in this large-scale study. Brain tissue (hypoxic and control) was then processed in the normal way as already described in Section 2.3 and membranes prepared. This surgical procedure is routinely used (Kendall et al, 1991).

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